PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 27 June 2000 (27.06.00)	
International application No. PCT/US99/26950	**Applicant's or agent's file reference** UA-338 PCT
International filing date (day/month/year) 16 November 1999 (16.11.99)	**Priority date** (day/month/year) 18 November 1998 (18.11.98)
Applicant JU, Lu-Kwang	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 April 2000 (21.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Olivia RANAIVOJAONA
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

Form PCT/ISA/210 (second sheet)(July 1992)
FILE COPY - DO NOT MAIL

International application No.
PCT/US99/26950

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 1/00, 39/00, 19/02. 19/44; C12N 1/20
US CL :435/41, 42, 74, 105, 253.3; 536/4.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/41, 42, 74, 105, 253.3; 536/4.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,501,966 A (GIANI ET AL) 26 MARCH 1996 (26/03/1996), see entire document, especially columns 3-9.	1-20,22-25, 27-38,41- 71 ---------- 21, 26, 39 and 40
Y	US 5,501,966A (GIANI ET AL) 26 MARCH 1996 (26/3/1995)see entire document, especially column 7-8	21, 26, 39 and 40
Y	VARMA AMIT et al. Stoichiometric Flux Balance Models Quantitatively Predict Growth and Metabolic By-Product Secretion in Wild-Type Escherichia coli W3110. Applied and Environmental Microbiology.October 1994, Vol 60, No .10, pages 3724-3731.	1-71

21, 26, 39, 40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 FEBRUARY 2000	Date of mailing of the international search report
Facsimile No. (703) 305-3230	Authorized officer AND Telephone No. PADMA BASKAR (703) 308-8886

INTERNATIONAL SEARCH REPORT
Form PCT/ISA/210 (continuation of second sheet)(July 1992)
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International application No.
PCT/US99/26950

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ATLAS RONALD. Hand Book of Microbiological Media : CRC press Ann Arbor, Pages 290-301.	21,26,39 and 40

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: RAY L. WEBER
RENNER, KENNER, GREIVE, BOBAK
TAYLOR & WEBER
16 TH FLOOR,FIRST NATIONAL TOWER
AKRON OH 44308

FILE COPY 408

WRITTEN OPINION

(PCT Rule 66)

Form PCT/IPEA/408 (cover sheet) (July 1998) DO NOT MAIL

Applicant's or agent's file reference UA-338 PCT		Date of Mailing *(day/month/year)* REPLY DUE within TWO months from the above date of mailing
International application No. PCT/US99/26950	International filing date *(day/month/year)* 16 NOVEMBER 1999	Priority date *(day/month/year)* 18 NOVEMBER 1998
International Patent Classification (IPC) or both national classification and IPC Please See Supplemental Sheet.		
Applicant THE UNIVERSITY OF AKRON		

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.
2. This opinion contains indications relating to the following items:
   - I [X] Basis of the opinion
   - II [ ] Priority
   - III [ ] Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application
3. The applicant is hereby invited to reply to this opinion.
   When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~
   How? By submitting a written reply, accompanied, where appropriate, by amend... ...ling to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and ...ule 66.4 *bis*.
   Also For an additional opportunity to submit amendments, see Rule 66.4. ...n the basis of this opinion. For the examiner's obligation to consider amendments and/or a... For an informal communication with the examiner, see Rule...
   If no reply is filed, the international preliminary examination report w...
4. The final date by which the international preliminary examination report must be established according to Rule 69.2...

Facsimile No. (703) 305-3230	...er AND Telephone No. BASKAR (703) 308-1235

WRITTEN OPINION
Form PCT/IPEA/408 (Box I) (July 1998)
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International application No.
PCT/US99/26950

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-22 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 23-33 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawing:
pages 1-6 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

WRITTEN OPINION
Form PCT/IPEA/408 (Box II) (July 1998)
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International application No.
PCT/US99/26950

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

WRITTEN OPINION

Form PCT/IPEA/408 (Box III) (July 1998)
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International application No.
PCT/US99/26950

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☐ claims Nos. _

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

WRITTEN OPINION

Form PCT/IPEA/408 (Box IV) (July 1998)
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International application No.
PCT/US99/26950

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1 not to invite the applicant to restrict or pay additional fees:

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

- ☐ all parts.
- ☐ the parts relating to claims Nos. .

WRITTEN OPINION

Form PCT/IPEA/408 (Box V) (July 1998)
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International application No.

PCT/US99/26950

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	21, 26, 39 AND 40	YES
	Claims	1-20, 22-25, 27-38,41-71	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-71	NO
Industrial Applicability (IA)	Claims	1-71	YES
	Claims	NONE	NO

2. citations and explanations

Claims 1-20, 22-25,27-38,41-71 lack novelty under PCT Article 33(2) as being anticipated by Giani et al 1966 (U.S.Patent 5,501,966). Giani et al disclose a process of the claimed invention in columns 3-9. Giani et al disclose a process for the biotechnological preparation of L-Rhamnose of Pseudomonas aeruginosa.

Claims 21,26,39, and 40 lack an inventive step under PCT Article 33(3) as being obvious over Giani et al 1966 (U.S.Patent 5,501,966)in view of Atlas Ronald . Giani et al teach using sodium nitrate in a process for the production of biological product such as L-Rhamnose. However, the prior art does not teach a process for the preparation of biological product from obligate anaerobes and facultative aerobes using sodium nitrite and fumarate in the culture medium . It would have been obvious to add nitrite instead of nitrate and fumarate to the culture medium as taught by Atlas Ronald so that suitable growth medium with an alternative oxidant source is provided for obligate anaerobes and facultative aerobes.

Claims 1-71 lack an inventive step under PCT Article 33(3) as being obvious over Varma et al. Varma et al teach Flux models using both aerobic and anaerobic bacteria for the production of by-products. The prior art teaches in pages 3726-3729 oxygen uptake rate, glucose uptake rate, biomass requirements for both aerobic and anaerobic bacteria.

---------- NEW CITATIONS ----------

NONE

WRITTEN OPINION

Form PCT/IPEA/408 (Box VI) (July 1998)
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International application No.
PCT/US99/26950

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication Date *(day/month/year)*	Filing Date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

International application No.
PCT/US99/26950

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

WRITTEN OPINION

Form PCT/IPEA/408 (Box VIII) (July 1998)
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International application No.

PCT/US99/26950

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

International application No.
PCT/US99/26950

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C12P 1/00, 39/00, 19/02. 19/44; C12N 1/20 and US Cl.: 435/41, 42, 74, 105, 253.3; 536/4.4

August 1967 • Volume 121, Number 2

ARCHIVES OF Biochemistry and Biophysics

ACADEMIC PRESS
New York and London

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 121, 304–310 (1967)

Concurrent Decrease of Enzymic Activities Concerned with the Synthesis of Coenzyme B_{12} and of Propionic Acid in *Propionibacteria*[1]

I. ARAVINDAKSHAN MENON AND DAVID SHEMIN

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received April 5, 1967; accepted April 5, 1967

Cells of *Propionibacterium shermanii* grown aerobically produce far less of the corrin ring of vitamin B_{12} than those cells grown anaerobically. The decreased amount of the corrin ring is in part explained by the diminished activities of enzymes concerned with the synthesis of the porphyrin-like ring of B_{12}. It has been found that the activity of δ-aminolevulinic acid synthetase in extracts of aerobically grown cells is about 60% of that found in extracts of anaerobically grown cells. Furthermore, the δ-aminolevulinic acid dehydrase activity in aerobic extracts is either not demonstrable, or only about 10% of that in extracts of anaerobically grown cells. The diminished concentration of coenzyme B_{12} in aerobically grown cells is reflected in the fatty acid composition of the fermentation products. Propionic acid, the predominant fatty acid among the products of the anaerobic fermentation, occurs in much smaller quantities as a product of aerobic fermentation. This is consistent with the well-known dependence of the methylmalonyl CoA-succinyl CoA isomerase reaction on coenzyme B_{12}. The addition of coenzyme B_{12} to extracts of aerobically grown cells restores the propionate-succinate conversion to only about 30–50% of that found in extracts of anaerobically grown cells. It appears, therefore, that along with the decreased activities of enzymes concerned with B_{12} synthesis, there is a concurrent decreased activity of an enzyme system which depends in part on coenzyme B_{12}. Since the cellular biotin concentration appears to be dependent on the amount of the biotin-dependent enzymes, the finding that extracts of aerobically grown cells contain less biotin than those of cells grown anerobically may be the result of the decreased amount of the functional biotin dependent enzyme.

Propionibacteria produce propionic acid, acetic acid, and carbon dioxide as major fermentation products. Evidence has been presented (1–6) that the formation of propionic acid from pyruvate by these organisms occurs by the following reactions:

$$\text{Succinyl-CoA} \rightleftarrows \text{Methylmalonyl-CoA} \quad (1)$$

$$\text{Methylmalonyl-CoA} + \text{Pyruvate} \rightleftarrows \text{Propionyl-CoA} + \text{Oxaloacetate} \quad (2)$$

$$\text{Oxaloacetate} + 4\text{H} \rightleftarrows \text{Succinate} + \text{H}_2\text{O} \quad (3)$$

$$\text{Succinate} + \text{Propionyl-CoA} \rightleftarrows \text{Succinyl-CoA} + \text{Propionate} \quad (4)$$

$$\text{Sum: Pyruvate} + 4\text{H} \rightleftarrows \text{Propionate} + \text{H}_2\text{O}$$

Furthermore, it has been demonstrated that methylmalonyl-CoA isomerase (Reaction 1) is coenzyme B_{12} dependent (2, 3, 5) and that transcarboxylation (Reaction 2) is a biotin dependent step (1, 2, 4). The participation of these coenzymes in the fermentation reactions of propionibacteria together with the observation that these organisms contain a comparatively high concentration of vitamin B_{12} and biotin derivatives is in harmony with the suggestion of Stadtman *et al.* (2) that the occurrence of

[1] This work was supported by grants from the United States Public Health Service (A-1101) and from the National Science Foundation (G-18712).

high concentrations of a vitamin or coenzyme in a microorganism may not be an artifact, but may reflect a unique role that the vitamin or its coenzyme plays in a major metabolic process carried out by the organism. It has been observed by several workers (7, 8), and confirmed by us, that propionibacteria synthesize the corrin moiety of vitamin B_{12} or Factor B under anaerobic conditions and that the highest yields of the cobamide can be obtained by first growing the organisms under anaerobic conditions followed by aerobiosis. Further, exceedingly small amounts of Factor B or cobamides are formed if the organism is grown only under aerobic conditions. It would seem that the decreased amount of cobalamins in the organism grown aerobically may be due to the repression or inactivation of enzymes involved in corrin ring synthesis and may be reflected in a changed metabolic pattern in the organism. Also, it appeared relevant to consider the possibility that as a consequence of the lack of synthesis of the cobalamins, either by some unknown direct effect by oxygen or by the repression of enzymes involved in the biosynthetic sequence of B_{12} there may be a reduction of the activity of the coenzyme-dependent enzymes catalyzing a biochemical reaction sequence even on the addition of the coenzyme to the test system.

We have compared the enzymic activities δ-aminolevulinic acid synthetase and δ-aminolevulinic acid dehydrase in extracts of cells grown anaerobically or aerobically. These enzymes catalyze the early steps of both porphyrin and corrin synthesis. The former enzyme catalyzes the synthesis of δ-aminolevulinic acid from succinyl-CoA and glycine, and the latter the synthesis of porphobilinogen from 2 moles of δ-aminolevulinic acid. Also, the activities of the enzymes catalyzing the propionate-succinate interconversion (see Eq. 5–8) and the fatty acid composition of the fermentation products were compared in these extracts. A preliminary report of the findings has been published (9).

EXPERIMENTAL PROCEDURE

Growth of bacteria. Propionibacterium shermanii (ATCC 9614 and 9615) was maintained in lactate-tryptone-yeast extract stab cultures and grown for 30 hours at 30° in tubes containing 10 ml of a lactate-tryptone-yeast extract medium. Twenty-five ml of these starter cultures were used to inoculate 1 liter of medium containing 30 gm glucose, 20 gm yeast extract, 0.01 gm $Co(NO_3)_2 \cdot 6H_2O$, and 0.005 M sodium potassium phosphate, pH 7.0 (10). The inoculated flasks were then incubated at 30° for 2–3 days under aerobic or anaerobic conditions. Under aerobic conditions filtered air was continuously bubbled through the culture medium at a rate of approximately 100 ml per minute through a gas-diffusing stone. The growth of the organisms under anaerobic conditions was accomplished by flushing the inoculated medium with nitrogen for 10 minutes and then tightly stoppering the flasks. The growth of the bacteria was followed by observing the optical density of the suspension in a Klett-Summerson colorimeter using a red filter (No. 66), and the acid produced was neutralized at intervals with 1 N ammonium hydroxide. The organisms were harvested by centrifugation toward the end of the exponential growth phase. The yield of cells was 2–4 gm wet weight per liter. The amounts and types of acid produced by the organisms under these two conditions of growth were determined in the supernatant solution. In experiments in which we wished to compare the amounts of vitamin B_{12} produced under different growth conditions, the cells were permitted to grow for 6 days in a medium containing corn steep liquor, glucose, and cobalt (8).

Cell-free extracts. The harvested cells were washed 3 times with 100 ml of 0.12 M KCl and then ground in the cold with an equal weight of alumina suspended in 5 ml of 0.12 M KCl. After grinding, 20 ml of ice-cold 0.12 M KCl was added and the cold suspension was stirred for 30 minutes. The suspension was centrifuged for 2 hours at 100,000*g* and the clear supernatant solution was assayed for vitamin B_{12}, biotin, and relevant enzymic activities.

Determinations. Protein was determined by the method of Lowry *et al.* (11). The amount of vitamin B_{12} in the cell extract was determined by a turbidometric bioassay with *Lactobacillies leichmanii* (ATCC 7830) essentially according to published procedure (12). Biotin was determined by both a yeast and a *Lactobacillus* assay; the yeast assay method was that of Genghaf *et al.* (13) and Hertz (14), and the *Lactobacillus* assay was that of Wright and Skeggs (15). The total biotin was determined on the cell-free extracts which were autoclaved for 1 hour at 18 pounds pressure after the addition of H_2SO_4 to a final concentration of 2 N. The free biotin was assayed on the cell-free extracts before hydrolysis, and

the combined biotin represents the difference between the total and free biotin (Table I). δ-Aminolevulinic acid synthetase activity was assayed by determining (16) the amount of δ-aminolevulinic acid formed from succinyl-CoA and glycine. Components of the assay systems are given in Table II. δ-Aminolevulinic acid dehydrase activity was assayed by determining the amount of formed porphobilinogin by Ehrlich's reagent (16). The components of the assay system are given in Table III. The conversion of propionate-1-^{14}C into the dicarboxylic acid was carried out by methods similar to those of Phares *et al.* (6) and Stadtman *et al.* (2). The components of the enzymatic system are given in Table IV. The incorporation of propionate into succinate was assayed by measuring the amount of ^{14}C fixed into the nonvolatile acid fraction. The incubation mixture was evaporated to dryness after the addition of 0.5 ml of 5 N HCl, and the residue was dissolved in water and evaporated to dryness again. Finally, the residue was dissolved in 1 ml of 0.5 N KOH, transferred to a planchet, and evaporated to dryness, and the radioactivity was determined. The nonvolatile acid was shown to be mostly succinic acid by the isolation from the reaction mixture after the above treatment of radioactive succinate (m.p. 189°) whose specific activity was equal to that of the nonvolatile acid fraction.

The determination of the amount and identity of the acids excreted in the medium by the cells grown aerobically and anaerobically was carried out in the following manner. The supernatant solution obtained after removal of the bacteria by centrifugation was extracted continuously with ether for 24 hours. The total amount of acid was determined by titration with a standard base on a suitable aliquot and the acids were identified by column chromatography. An aliquot of the ether solution was extracted with a sodium bicarbonate solution, and the aqueous solution, after acidification with H_2SO_4, was placed on a Celite column (10 × 17.5 cm) containing 0.02 N H_2SO_4. The acids were eluted by the successive passage of 100 ml of $CHCl_3$, 100 ml of $CHCl_3$ containing 5% *n*-butanol, and 100 ml of $CHCl_3$ containing 10% *n*-butanol. Propionic acid was eluted by the $CHCl_3$, acetic acid by the chloroform containing 5% *n*-butanol, and succinic acid by the chloroform containing 10% *n*-butanol; their elution patterns were similar to that obtained with known mixtures of the acids. The compounds were located in the eluate fractions (10 ml) by titration with a standard solution of NaOH, and the amounts of each were determined.

Materials. Coenzyme A was purchased from Sigma Chemical Company and δ-aminolevulinic acid from Mann Research Laboratories; co... steep liquor was a gift from the Corn Industri... Research Foundation, and coenzyme B_{12} was ... gift from Dr. D. Perlman of the Squibb Institut... Succinyl-CoA was prepared by the method ... Simon and Shemin (17). Sodium propionate-1-^{14}C ... purchased from New England Nuclear Corpor... tion, was diluted approximately fiftyfold wi... nonradioactive propionate; the specific radio... activity of the diluted sample was 0.046 mC... mmole.

RESULTS

Synthesis of the corrin structure. We have found that *Propionibacterium shermanii* grown anaerobically for 3 days and then aerobically for 3 days produced about 5–10 mg of vitamin B_{12} per liter, but the cells grown aerobically for 6 days synthesized comparatively minute quantities of B_{12} (0.014–0.03 mg/liter). This finding is in agreement with those of previous investigators (7, 8), whose results indicate that the Factor B is synthesized by propionibacterium under anaerobic conditions and that the completion of the synthesis of the vitamin occurs under aerobic conditions. The yield of vitamin B_{12} from the organism grown anaerobically followed by aerobiosis is much larger than from those grown only under anaerobic conditions (7, 8).

Biotin content. It can be seen from Table I that the total biotin content of the organism grown aerobically is considerably less than that found in the organisms grown anaerobically.

δ-Aminolevulinic acid synthetase. Table

TABLE I

AMOUNTS OF BIOTIN IN EXTRACTS OF *Propionibacterium shermanii* GROWN UNDER ANAEROBIC OR AEROBIC CONDITIONS

	Concentration of biotin (mμg/mg of protein)			
	Anaerobic growth		Aerobic growth	
	Yeast assay	Lactobacillus assay	Yeast assay	Lactobacillus assay
Total biotin[a]	81	110	26	30
Free biotin[b]	12	9	4	3
Combined biotin[c]	69	101	22	27

[a] Biotin determined after hydrolysis.
[b] Biotin determined before hydrolysis.
[c] Obtained by difference.

lann Research Laboratories; corn was a gift from the Corn Industries ındation, and coenzyme B_{12} was a D. Perlman of the Squibb Institute. was prepared by the method of emin (17). Sodium propionate-1-^{14}C, m New England Nuclear Corpora- uted approximately fiftyfold with propionate; the specific radio- ıe diluted sample was 0.046 mC/

RESULTS

f the corrin structure. We have *Propionibacterium shermanii* ›bically for 3 days and then r 3 days produced about 5–10 n B_{12} per liter, but the cells :ally for 6 days synthesized minute quantities of B_{12} ıg/liter). This finding is in h those of previous investiga- ıose results indicate that the synthesized by propionibac- :naerobic conditions and that of the synthesis of the vita- der aerobic conditions. The B_{12} from the organism grown)llowed by aerobiosis is much m those grown only under tions (7, 8).

. It can be seen from Table I otin content of the organism ly is considerably less than ıe organisms grown anaero-

'ic acid synthetase. Table

TABLE I

OTIN IN EXTRACTS OF *Pro- shermanii* GROWN UNDER R AEROBIC CONDITIONS

Concentration of biotin (mμg/mg of protein)			
Anaerobic growth		Aerobic growth	
Yeast assay	Lacto- bacillus assay	Yeast assay	Lacto- bacillus assay
81	110	26	30
12	9	4	3
69	101	22	27

d after hydrolysis.
d before hydrolysis.
:rence.

TABLE II

ACTIVITY OF δ-AMINOLEVULINIC ACID SYNTHETASE IN EXTRACTS OF *Propionibacterium shermanii* GROWN ANAEROBICALLY AND AEROBICALLY

The assay system contained 1 μmole of succinyl-CoA, 0.5 μmole pyridoxal 5-phosphate, 50 μmoles glycine, 3 μmoles EDTA, 0.5 μmole of mercapto-ethanol, and the bacterial extract in a total volume of 3 ml. Each of the determinations was carried out with 1.5 and 3 mg of protein. The reaction mixture was incubated at 34° for 20 minutes.

Expt. No.	δ-Aminolevulinic acid (mμmole/mg protein)	
	Anaerobic growth	Aerobic growth
1	16	10
2	20	14
3	13	6
4	16	9
5	11	7
6	12	7
7	23	13
8	23	13
9	24	12

TABLE III

ACTIVITY OF δ-AMINOLEVULINIC ACID DEHYDRASE IN EXTRACTS OF *Propionibacterium shermanii* GROWN ANAEROBICALLY AND AEROBICALLY

The incubation mixture contained 10 μmoles δ-aminolevulinate, 10 μmoles GSH, 1.0 ml of 0.1 M sodium phosphate-carbonate buffer, pH 8.0, and bacterial extracts in a volume of 2 ml. Each of the determinations was carried out with 1.5 and 3.0 mg of protein. The reaction mixture was incubated for 20 minutes at 34°.

Expt. No.	Porphobilinogen (mμmoles/mg of protein)	
	Anaerobic	Aerobic
1	20	—[a]
2	18	—[a]
3	15	—[a]
4	20	—[a]
5	21	—[a]
6	16	2[b]
7	11	2[b]
8	43	1[b]
9	21	2[b]

[a] Activity too small to be measured. OD less than 0.01 above blank.

[b] 2 mμmoles corresponds to an OD of approximately 0.02 above blank of 0.02.

II gives several typical values for δ-aminolevulinic synthetase activity of extracts of cells grown anaerobically and aerobically. It can be seen that the extracts of the aerobic organism had about 60% of that found in the extracts of anaerobically grown cells.

δ-Aminolevulinic acid dehydrase. Table III compares the activities of δ-aminolevulinic

TABLE IV

CONVERSION OF PROPIONATE-1-^{14}C INTO DICARBOXYLIC ACIDS BY EXTRACTS OF *Propionibacterium shermanii* GROWN ANAEROBICALLY OR AEROBICALLY

The complete system contained potassium succinate, 25 μmoles; sodium propionate-1-^{14}C- (0.046 μC/μmole), 10 μmoles; acetyl-CoA, 0.1 μmole; sodium maleate buffer (pH 6.5), 10μmoles; sodium-magnesium versenate, 5 μmoles; cysteine, 5 μmoles; and bacterial extracts, 1 mg protein. The amount of supplements added in the experiments indicated in the Table were: ATP, 5 μmoles; biotin, 0.4 μmole; coenzyme B_{12}, 0.002 μmoles; and boiled extract of anaerobically grown cells (0.2 ml of an extract made from 0.5 gm wet weight of cells per ml). The mixtures, total volume of 2 ml, were incubated at 38° for 1 hour.

Incubation system	^{14}C Activity of succinic acid[a] (cpm)	
	Anaerobic extract	Aerobic extract
Complete system	5100	200
+ Coenzyme B_{12}	6200	2000
+ Boiled extract	6200	1800
+ Biotin	5200	70
+ ATP	5300	400
+ Coenzyme B_{12} + biotin	6100	1900
+ Coenzyme B_{12} + ATP	7600	2800
+ Biotin + ATP	5600	400
+ Coenzyme B_{12} + biotin + ATP	7600	2900
Complete system	8400	90
+ Coenzyme B_{12}	10,300	3200
+ Boiled extract	7900	1900
Complete system	5000	20
+ Coenzyme B_{12}	5800	3200
+ Boiled extract	5600	2800

[a] Nonvolatile acid (see text). The reported ^{14}C activities in the nonvolatile fraction are the values found less the activity found at zero time in each of the flasks. The radioactivity at zero time ranged from 220 to 280 cpm.

dehydrase of extracts of cells grown aerobically and anaerobically. It can be seen that whereas the extracts of cells grown anaerobically formed 20–40 mμmoles of porphobilinogen per milligram of protein, the extracts of the cells grown aerobically were at most 10% as active, and in many preparations no activity could be demonstrated.

Propionate-succinate interconversion. The conversion of propionate-^{14}C into succinate is readily explained by the following reaction mechanism (2, 18).

$$\text{Acetyl-CoA} + \text{Succinate} \rightleftarrows \text{Succinyl-CoA} + \text{Acetate} \quad (5)$$

$$\text{Succinyl-CoA} \xrightleftharpoons{\text{Coenzyme } B_{12}} \text{Methylmalonyl-CoA} \quad (6)$$

$$\text{Methylmalonyl-CoA} + \text{Biotin-enzyme} \rightleftarrows CO_2\text{-Biotin-enzyme} + \text{propionyl-CoA} \quad (7)$$

$$\text{Propionyl-CoA} + \text{Acetate} \rightleftarrows \text{Acetyl-CoA} + \text{Propionate} \quad (8)$$

$$\text{Sum: Succinate} + \text{Biotin-enzyme} \rightleftarrows CO_2\text{-Biotin-enzyme} + \text{Propionate}$$

Table IV lists the radioactivities of the dicarboxylic acid synthesized from propionate-1-^{14}C in extracts of cells grown anaerobically or aerobically. It can be seen that the extracts of aerobically grown cells are only about 1–4% as active as those grown anaerobically. This low activity was due to a great extent to the lack of coenzyme B_{12} in the extract of aerobically grown cells, for addition of this coenzyme or of boiled extract of anaerobically grown cells to these extracts increased the activity to 30–50% of that found in the extract of anaerobic grown cells. However, full activity was not restored. The addition of biotin did not increase the activity in the extracts of aerobic grown cells. It would seem that the biotin concentration, although smaller, was not limiting.

Comparison of acid excretion patterns. It was found that the cells grown aerobically produced less acid per gram of cells than those grown anaerobically, and that the fatty acid composition of the fermentation products was strikingly different (Fig. 1). Cells grown aerobically to about the same optical density as those grown anaerobically produced about 6–8 meq of acid per gram of wet weight of cells, whereas the anaerobic

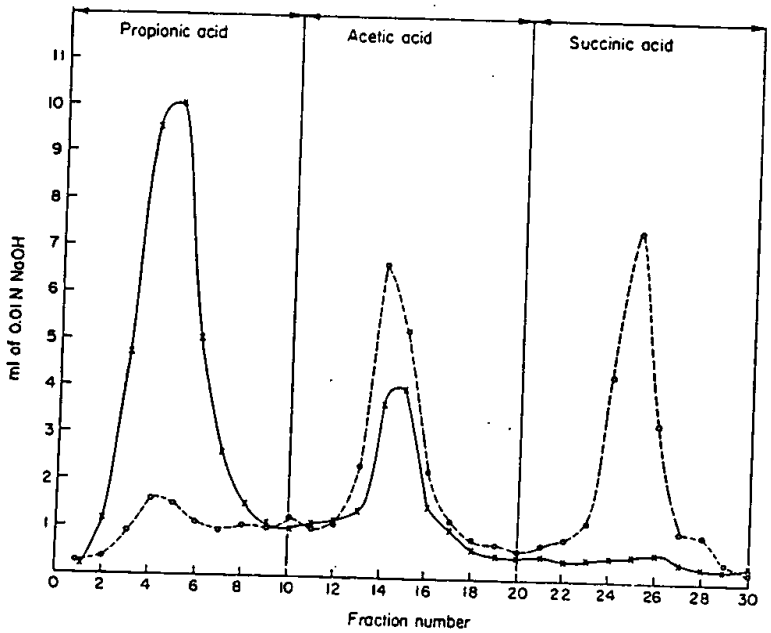

FIG. 1. Pattern of acids formed by Propionibacteria grown under anaerobic condition (——) or under aerobic conditions (– – –). Approximately equal amounts of total fatty acids were used to obtain the fatty acid excretion pattern.

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growth produced 20–24 meq of acid per gram of wet weight of cells. It can be seen from Fig. 1 that the acid pattern is also different. The anaerobically grown cells produced mainly propionic acid (70–75%) and smaller amounts of acetic acid (20–25%), whereas the aerobically grown cells produced largely succinic acid (43–49%), acetic acid (36–46%), and small amounts of propionic acid (10–15%).

DISCUSSION

It has been observed among different organisms that aerobiosis both induces or represses porphyrin synthesis. In most instances the oxygen effect on porphyrin synthesis is in the direction of the changed metabolic requirements of the cell. For example, the cytochrome concentration in the yeast, *Saccharomyces cerevesiae*, is many fold higher in cells cultivated under aerobic conditions than in those grown anaerobically (19). On the other hand, the concentration of bacteriochlorophyll in the photosynthetic organisms, *Rhodopseudomonas spheroides*, is far less in the organism grown aerobically than in those cells grown under anaerobic photosynthetic conditions (20). Similarly, as shown in this paper, the amount of the corrin moiety of cobamide in propionic bacteria is influenced by oxygen and anaerobiosis. Propionic acid bacteria grown on glucose under anaerobic conditions utilize pyruvate as a hydrogen acceptor (see Eqs.1–4). This reduction, as has been recently elucidated, occurs via a sequence of reactions in which cobamide coenzyme and biotin are required. However, when the organism is grown under aerobic conditions, oxygen, rather than pyruvate, is the electron acceptor. Under these conditions the organism synthesizes only minute quantities of the porphyrin-like structure of the cobamide. This may be responsible for the changed fatty acid composition of the fermentation products formed by the organism grown under aerobic conditions.

The effect of oxygen may be due to a primary event on some enzymes concerned with porphyrin synthesis or may be a direct repression of enzymes involved in porphyrin synthesis. Although nothing is known of the mechanism of oxygen inhibition of porphyrin synthesis, the almost complete absence of δ-aminolevulinic acid dehydrase activity and the partial reduction of δ-aminolevulinic acid synthetase activity may account for the markely reduced cobamide synthesis. The decrease of δ-aminolevulinic acid synthetase and dehydrase activities has been noted earlier in *Rhodopseudomonas spheroides* and has been attributed to enzyme repression (21, 22), but the extent of the repression appeared not to be sufficient to account for the almost complete lack of synthesis of bacteriochlorophyll noted in these organisms grown aerobically (22). The reduction of δ-aminolevulinic acid dehydrase activity in *P. shermanii* by oxygen, however, appears sufficiently severe to be consistent with the marked inhibition of cobamide synthesis. The δ-aminolevulinic acid dehydrase activity, in extracts grown aerobically, was in many cases not demonstrable and at best contained only about 10% of that found in extracts of anaerobically grown cells. The residual activity appears to be sufficient to meet the need for the synthesis of heme compounds necessary for electron transport and can account for the very small amount of coenzyme B_{12}. The excretion of small amounts of propionic acid under aerobic growth conditions can also be taken as evidence for the continued synthesis of small amounts of porphyrin.

It appears that the change in dehydrase activity in cells grown aerobically is not due to the accumulation of an inhibitory compound, for extracts of aerobically grown cells do not inhibit δ-aminolevulinic acid dehydrase when added to extracts of cells grown anaerobically.

It appeared likely that *P. shermanii* grown aerobically may not readily be capable of interconverting propionate and succinate since methylmalonyl - CoA/succinyl - CoA isomerase (Reaction 1) is coenzyme B_{12}-dependent. This is supported by the data in Table IV. However, the lack of incorporation of propionate-^{14}C into succinate is not wholly due to the lack of coenzyme B_{12}, for the conversion is not fully restored on addition of the coenzyme to extracts of aerobically grown cells. The aerobic extracts are

only about 30–50% as active after the addition of coenzyme B_{12} or of boiled extracts of anaerobically grown cells. It therefore appears that along with the reduced activities of enzymes concerned with B_{12} synthesis, there is a concurrent decrease of activity of enzymes or of an enzyme participating in a metabolic sequence and which contains a coenzyme B_{12}-dependent enzyme. It has not as yet been demonstrated that the decreased enzymic activity is specific for the B_{12}-dependent enzyme, nor limited to this enzyme. However, the decreased enzymic activity appears to include the biotin-dependent reaction. This is based on the reasonable assumption that the concentration of bound biotin is dependent on the amount of biotin-dependent enzymes.

It would seem, therefore, that along with the reduction of enzymic activities concerned with the synthesis of coenzyme B_{12}, there appears to be a concurrent decrease of activity in an enzyme system whose function is dependent on this coenzyme. Whether the synthesis of enzymes concerned in the succinate-propionate conversion are regulated by their coenzymes is not known. However, it appears that the synthesis of pyruvate decarboxylase is regulated by its coenzyme, thiamine (23), that globin synthesis is regulated by heme (24–27), and that pyridoxin induces an increase in tyrosine transaminase (28).

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Transcriptional regulation and energetics of alternative respiratory pathways in facultatively anaerobic bacteria

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Abstract

The facultatively anaerobic *Escherichia coli* is able to grow by aerobic and by anaerobic respiration. Despite the large difference in the amount of free energy that could maximally be conserved from aerobic versus anaerobic respiration, the proton potential and $\Delta G'_{Phos}$ are similar under both conditions. O_2 represses anaerobic respiration, and nitrate represses fumarate respiration. By this the terminal reductases of aerobic and anaerobic respiration are expressed in a way to obtain maximal H^+/e^- ratios and ATP yields. The respiratory dehydrogenases, on the other hand, are not synthesized in a way to achieve maximal H^+/e^- ratios. Most of the dehydrogenases of aerobic respiration do not conserve redox energy in a proton gradient whereas the enzymes from anaerobic respiration do so. Thus transcriptional regulation of the respiratory pathways by electron acceptors has multiple effects on cellular energetics. The transcriptional regulation in response to O_2 is effected by two transcriptional regulators, ArcA/B (aerobic respiratory control) and FNR (fumarate nitrate reductase regulator). FNR contains an O_2-sensitive $[4Fe\text{–}4S]^{2+}$ cluster in the sensory domain and is converted to the transcriptional inactive state in the presence of (cytoplasmic) O_2. © 1998 Elsevier Science B.V.

Keywords: Aerobic and anaerobic respiration; Proton potential; Transcriptional regulation; Regulation of energetics; Fumarate nitrate reductase regulator; O_2-sensing

1. Introduction

Facultatively anaerobic bacteria like *Escherichia coli* are able to use nitrate, fumarate and dimethylsulfoxide (DMSO) as acceptors for respiration if no oxygen is available, or to gain energy by fermentation. The switch from aerobic to anaerobic catabolism has many consequences on cellular energetics and requires the presence of O_2 and nitrate sensitive transcriptional regulators to adapt the expression of the respective genes [7,10,11,20,24–26]. In *E. coli* two O_2-responsive transcriptional regulators are known, ArcA/B (*a*erobic *r*espiratory *c*ontrol) [11] and FNR (*f*umarate *n*itrate reductase *r*egulator) [10,20,24,25], which control expression of the respective genes in response to O_2. The switch from aerobic to anaerobic respiration (and fermentation) has important consequences on cellular energetics and ATP yields, since the free energy differences for the anaerobic respiratory chains are distinctly lower than those for the aerobic [10,24–26]. It turned out that the synthesis of the terminal reductases is regulated in a way to synthesize the enzymes with maximal ATP yields under the respective growth condition [10,11,24]. The synthesis of alternative respiratory dehydrogenases, on the other hand, often is not

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regulated such as to obtain maximal ATP yields [22,25]. Thus understanding the rationale of transcriptional regulation by environmental signals like O_2 and nitrate is important for the understanding of cellular energetics.

2. Energetics of growth by aerobic and anaerobic respiration in facultatively anaerobic bacteria

In the absence of oxygen, less energy can be produced from oxidation of carbon sources during anaerobic respiration due to the different redox potentials of the terminal acceptors [22,24,25]. The free energy ($\Delta G^{0\prime}$) for glucose oxidation with O_2, for example, is up to 13-fold higher than for fermentation or anaerobic respiration (Table 1). Similarly, $\Delta G^{0\prime}$ for respiration with NADH decreases from −233 kJ/mol NADH in aerobic respiration to −67 kJ/mol in fumarate respiration [10,24,25]. Nevertheless, the energetic parameters of *E. coli* cells do not change largely upon aerobic/anaerobic transition (Table 1) [23]. Under steady state conditions of aerobic and anaerobic respiration and fermentation, the phosphorylation potential stays constant at 47 kJ/mol. The proton potential of −160 mV during aerobic respiration decreases only slightly during anaerobic respiration irrespective of the midpoint potential of the acceptor. Only in fermentation a more significant decrease in Δp is observed (Table 1). Due to the very similar energetic situation processes depending on Δp such as ATP-synthesis or transport of solutes across membranes can function in a similar way under conditions of aerobic and anaerobic respiration [23].

For the Δp values given in Table 1 the H^+/ATP ratios for ADP phosphorylation are in the range from 3.1 to 3.6 for aerobic and anaerobic respiration, compatible with a H^+/ATP ratio of 4 found for plant type F_0F_1 ATPase [28]. A supposed decrease of Δp to about −100 mV in anaerobic respiration of *E. coli* as suggested earlier, requires an increase of the H^+/ATP ratio to a value of 5 for ADP phosphorylation in anaerobic respiration, which is unlikely. Thus it is obvious that *E. coli* maintains $\Delta G'_{Phos}$ and Δp at rather constant levels during aerobic and anaerobic respiration despite large differences in ΔE of the respiratory chains. The same applies also to the anaerobic bacterium *Wolinella succinogenes*, which is able to grow by respiration with electropositive (nitrate, $E^{0\prime}=+420$ mV, or fumarate, $E^{0\prime}=+30$ mV) and electronegative acceptors (polysulfide, $E^{0\prime}=-260$ mV). With both types of acceptors (and H_2 or formate as the donor) Δp maintains constant at about −170 mV [14,19].

3. Synthesis of terminal reductases, but not of the respiratory dehydrogenases is optimized for maximal ATP yields

In *E. coli* and other facultatively anaerobic bacteria the synthesis of respiratory enzymes is regulated at the transcriptional level by regulators responding to O_2 and nitrate [10,11,24–27]. The oxidases and terminal reductases are expressed in a hierarchical way, i.e., oxygen represses fermentation and anaerobic respiration with nitrate or fumarate, and nitrate prevents fermentation and fumarate respiration by repression of the respective structural genes. The

Table 1
Energetic parameters of *E. coli* for growth by aerobic and anaerobic respiration and by fermentation

Acceptor for respiration	$\Delta G^{0\prime}$ (kJ/mol Gluc)	$E^{0\prime}$ (mV)	$\Delta G'_{Phos}$* (kJ/mol)	Δp* (mV)	m_{mle} (H^+/ATP)
O_2	−2830	+820	47.7	−160	3.1
Nitrate	−858	+420	46.2	−140	3.3
DMSO	−650	+160	47.2	−137	3.6
Fumarate	−550	+30	47.6	−145	3.4
–(Fermentation)	−218	–	46.5	−117	–

* Measured with glycerol as the C-source, except for fermentation (glucose).
The $\Delta G^{0\prime}$ values refer to growth on glucose and the respective acceptors [24], the values for $\Delta G'_{Phos}$ and Δp are taken from Ref. [23]. The number of H^+ required for ATP synthesis by ATP-synthase (m_{mle}) was calculated from $\Delta G'_{Phos} \leq m \cdot F \cdot \Delta p$ [23].

enzymes associated with the corresponding respiratory chains have different H^+/e^- ratios and amount to 2, 1, and 0 for quinol oxidase *bo* (Cyo), nitrate reductase (NarG) and fumarate reductase (Frd), respectively (see Refs. [7,25]). The ATP yields are thus highest for the oxidases, intermediate for nitrate reductase, and lowest for fumarate reductase which could be the regulatory rationale for the observed hierarchy in regulation. In other bacteria, however, electron acceptors with high ATP yields are not used preferentially. In *W. succinogenes* the most electronegative acceptor (polysulfide) with the lowest ATP yields, represses nitrate and fumarate respiration which both give higher ATP yields [15].

Many of the respiratory dehydrogenases of *E. coli* are transcriptionally regulated by oxygen and nitrate, too, and in aerobic and anaerobic respiration different dehydrogenases are used [5,9,22,25,27]. Most of the dehydrogenases which are synthesized under aerobic conditions apparently are not able to couple the redox reaction to the generation of a proton potential, although the redox reactions could supply sufficient free energy for H^+ translocation. This applies to glycerol-3-P (GlpD), NADH (Ndh), lactate (Dld, LctD), D-amino acid (DadA) dehydrogenases and pyruvate oxidase (PoxB) (Fig. 1) (see Ref. [25]). On the other hand, dehydrogenases which are mainly involved in anaerobic respiration, like NADH (NuoA-N), glycerol-3-P (GlpA) and formate (FdnG) dehydrogenases or hydrogenase (HybABC) are known, or supposed, to couple the redox reaction to H^+ translocation (Fig. 1) [4,22,25]. This principle becomes most obvious for the isoenzymes of dehydrogenases which are present in the bacteria (Fig. 1): the coupling isoenzymes (NuoA-N and presumably GlpA) operate in anaerobic respiration, the noncoupling enzymes (Ndh and GlpD) are the major enzymes in aerobic respiration [6,22,25]. The requirement for the coupling enzyme in (anaerobic) fumarate or DMSO respiration is obvious, since the enzyme provides the only site for H^+ translocation. Under an energetic point of view, the NADH:quinone oxidoreductase reaction could be coupled to energy conservation in aerobic respiration as well, and the same applies to the use of the glycerol-3-phosphate dehydrogenase isoenzymes under oxic and anoxic conditions. Thus, it appears that most of the dehydrogenases of aerobic respiration do not conserve redox energy in a proton gradient, whereas most dehydrogenases of anaerobic respiration do so [25,27]. This causes H^+/e^- ratios or ATP yields in aerobic respiration which are distinctly below the values which could be achieved (Table 2) [6,25]. This indicates that high metabolic flux rates which are stimulated by low coupling coefficients, are important in aerobic growth when ATP yields are not limiting. A similar principle can be observed for other pathways of *E. coli*, too, which are not expressed in sufficient amounts to achieve optimal ATP yields [17].

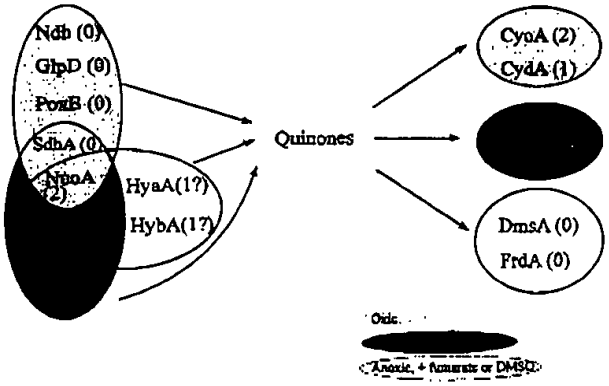

Fig. 1. Dehydrogenases and terminal reductases of aerobic and anaerobic respiration of *E. coli*. The figure gives the conditions (i.e., presence of electron acceptors) for the synthesis of the respiratory dehydrogenases and terminal reductases of the respective respiratory chains. The data are obtained from expression studies, analysis of mutants and measurement of enzyme activities. For any condition only the major enzyme present is considered. For each of the enzymes the H^+/e^- ratio is given in brackets. For more details see Ref. [25].

Table 2
Variation in H^+/e^- ratios for aerobic and fumarate respiration in *E. coli* (NADH as donor) by the use of alternative isoenzymes

Reaction	Enzymes	H^+/e^-	Comment
NADH→O_2	Nuo+Cyo	2+2=4	
	Nuo+Cyd	2+1=3	
	Ndh+Cyo	0+2=2	Major path
	Ndh+Cyd	0+1=1	
NADH→fumarate	Nuo+Frd	2+0=2	Major path
	Ndh+Frd	0+0=0	

The isoenzymes are NADH dehydrogenase I (Nuo) or II (Ndh), or quinol oxidases *bo* (Cyo) and *bd* (Cyd). The H^+/e^- ratios give the ratios for the individual enzymes and the complete path. For references, see Ref. [25].

4. Regulators and signals controlling the synthesis of respiratory enzymes in response to electron acceptors

Transcriptional regulation of the genes of aerobic and anaerobic respiration is effected mainly in response to O_2 and nitrate, but also to the type of the C-source and the growth phase. For the O_2 regulated genes defined O_2 tensions for half-maximal expression ($pO_{0.5}$) can be determined in an oxystat [1,2,26]. The *sdh* genes encoding succinate dehydrogenase of aerobic respiration are expressed efficiently only at high oxygen tensions (above 5 mbar O_2), whereas the genes or metabolic pathways of microaerobic respiration (between 1 to 5 mbar), anaerobic respiration (below 5 mbar) or fermentation (below 1 mbar) are expressed or functional at distinctly lower oxygen tensions. Thus the corresponding metabolic systems are functional in succession with decreasing pO_2 (aerobic respiration> microaerobic respiration>anaerobic respiration> fermentation).

Regulation by O_2 is effected by the O_2-sensing transcriptional regulators FNR and ArcA/B [11,20], regulation by nitrate by the sensor-regulators NarX/L and NarP/Q [21]. The latter are two-component regulatory systems consisting of a membraneous sensory kinase (ArcB, NarX, NarQ) and a cytoplasmic response regulator (ArcA, NarL, NarQ) (for reviews see Refs. [11,21]). The second O_2-sensor, FNR, is a 'one-component' sensor-regulator consisting of a sensory and of a regulatory domain within the same protein [20,26]. The protein is located in the cytoplasm of the bacteria and is assumed to react there directly with molecular oxygen. It has been shown that under aerobic and microaerobic conditions the cytoplasm of bacteria is rich in O_2 [29] due to the rapid diffusion of oxygen and the small cell dimensions [1,26,27]. Thus there is sufficient O_2 present in the cytoplasm for direct reaction with FNR. Only at very low external O_2 tensions ($pO_2<1$ mbar) the cytoplasm might become anoxic in accordance with the relevant regulatory O_2 tensions of FNR.

The sensory domain of FNR consists of a Fe–S cluster which is of the $[4Fe–4S]^{2+}$ type under anoxic conditions [3,8,12,16,26,27]. Then the protein is in the active state and activates or represses target genes. The cluster is liganded by four Cys-residues, three of which (Cys_{20}, Cys_{23}, Cys_{29}) are located in the N-terminal end, the fourth (Cys_{122}) in the central part of the protein. It is suggested that the cytoplasmic oxygen reacts with FNR by direct interaction [1,26,27,30]. In vitro, the Fe–S cluster is converted to a $[2Fe–2S]^{2+}$ cluster by oxygen, resulting in FNR inactivation [13]. The significance of a $[3Fe–4S]^{+}$ cluster which was observed after incubation of FNR with oxygen is not clear [8,12]. After prolonged exposure to oxygen, the Fe–S cluster is destroyed. Reassembly of the [4Fe–4S] cluster might require cellular proteins such as the NifS-like protein of *E. coli* [8,12,27].

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Redundancy of Aerobic Respiratory Chains in Bacteria? Routes, Reasons and Regulation

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ABSTRACT

Bacteria are the most remarkable organisms in the biosphere, surviving and growing in environments that support no other life forms. Underlying this ability is a flexible metabolism controlled by a multitude of environmental sensors and regulators of gene expression. It is not surprising, therefore, that bacterial respiration is complex and highly adaptable: virtually all bacteria have multiple, branched pathways for electron transfer from numerous low-potential reductants to several terminal electron acceptors. Such pathways, particularly those involved in anaerobic respiration, may involve periplasmic components, but the respiratory apparatus is largely membrane-bound and organized such that electron flow is coupled to proton (or sodium ion) transport, generating a protonmotive force. It has long been supposed that the multiplicity of pathways serves to provide flexibility in the face of environmental stresses, but the existence of apparently redundant pathways for electrons to a *single* acceptor, say dioxygen, is harder to explain. Clues have come from studying the expression of oxidases in response to growth conditions, the phenotypes of mutants lacking one or more oxidases, and biochemical characterization of individual oxidases. Terminal oxidases that share the essential properties of substrate (cytochrome *c* or quinol) oxidation, dioxygen reduction and, in some cases, proton translocation, differ in subunit architecture and complement of redox

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centres. Perhaps more significantly, they differ in their affinities for oxidant and reductant, mode of regulation, and inhibitor sensitivity; these differences to some extent rationalize the presence of multiple oxidases. However, intriguing requirements for particular functions in certain physiological functions remain unexplained. For example, a large body of evidence demonstrates that cytochrome *bd* is essential for growth and survival under certain conditions. In this review, the physiological basis of the many phenotypes of Cyd^- mutants is explored, particularly the requirement for this oxidase in diazotrophy, growth at low protonmotive force, survival in the stationary phase, and resistance to oxidative stress and Fe(III) chelators.

1. INTRODUCTIO

1.1. Scope

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1. INTRODUCTION

1.1. Scope

'*There is reason in the roasting of eggs*', 1659, J. Howell, *Proverbs* (English)

The aim of this review is to summarize and reassess our knowledge of branched, aerobic respiratory systems in bacteria. In effect, this brief encompasses the vast majority of the respiratory chains known in bacteria, since one of the distinguishing features of their respiratory metabolism appears to be a remarkable flexibility and modular construction. This allows individual components from the major module types (i.e. dehydrogenases, quinones, various cytochromes, and terminal oxidases and reductases) to be utilized in combinations that best enable the organism to respire and conserve energy under the prevailing, but changeable, environmental conditions. A comprehensive review on bacterial respiratory systems would now require several volumes, such has been the progress in this field over the past few decades. We try to focus attention on newer developments, current opinions, and perhaps overlooked aspects. We have selected respiratory chains in a relatively small number of bacteria that illustrate the principle of branching. Specifically we ask the question: why the multiplicity of respiratory routes? We examine whether, for example, when more than one oxidase is present, there appears to be, from a teleological perspective, an 'advantage' in use of one oxidase in preference to another. We also review recent work that reveals that oxidases may be non-essential for respiratory electron transfer and energy conservation but essential for some other role, such as ion transport, dioxygen scavenging, or redox homeostasis. Finally, we examine the small number of cases where simple unbranched respiratory chains do operate and question whether this constrains bacterial survival to a more restricted set of possible environments.

One of the most obvious examples of branching in bacterial respiratory chains is that which allows electron flux to either dioxygen or, in its absence, an alternative electron acceptor (anaerobic respiration). On the grounds that such branching requires little explanation (to take again, as will be common in this review, a teleological view), we do not consider anaerobic respiration in detail nor the mechanisms that allow its operation when dioxygen is unavailable. Instead we focus on aerobic respiration – the series of coupled oxidation and reduction reactions that result in the transfer of electrons from an appropriate electron donor (such as a reduced coenzyme) to dioxygen. It provides the ability to conserve energy in the form of adenosine triphosphate (ATP) or perform energy-demanding processes (such as solute transport or motility) through generation of a protonmotive force. Our definition of aerobic

respiration includes (as it does in multicellular organisms) the transport and storage of dioxygen.

1.2. Routes to Dioxygen, the Ultimate Electron Acceptor

In terms of energy conservation, respiration-coupled oxidative phosphorylation via the transmembrane protonmotive force is substantially more efficient than fermentation, allowing faster growth and attainment of higher yields of biomass per mole of energy substrate used. Aerobic respiration is identical in principle to anaerobic respiration, except that, in the latter, an electron acceptor other than dioxygen is used (Poole, 2000).

In aerobic respiration, an electron donor is oxidized and electrons derived from this oxidation are transferred sequentially through electron carriers and used to reduce dioxygen. For the complete reduction of dioxygen to water, which requires four electrons, the standard redox potential ($E^{\circ\prime}$) of the couple is +815 mV. Thus, use of dioxygen as an electron acceptor is more likely to result

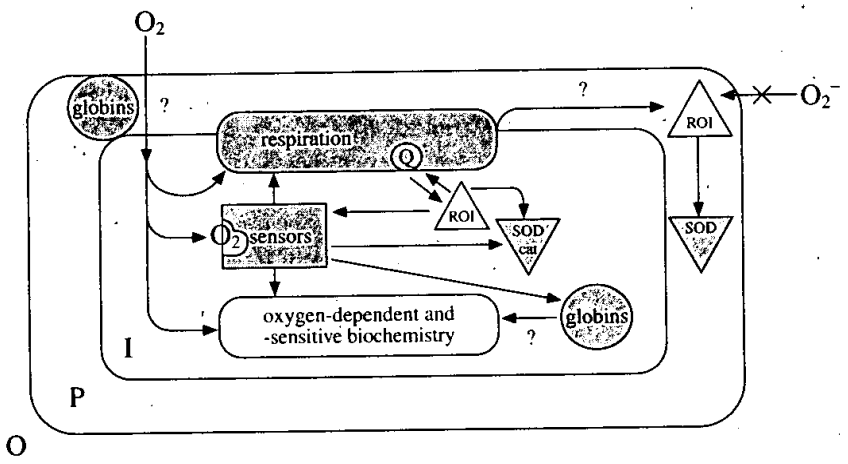


Figure 1 Dioxygen as friend and foe. Dioxygen readily permeates from the outside (O) to the cell interior (I) through the outer and cytoplasmic bacterial membranes, and the intervening periplasm (P), although globins have been suggested to facilitate the process. Dioxygen is reduced primarily to water in respiration, but is also required for dioxygen-dependent biochemistry such as oxygenase function. Dioxygen is sensed by several systems, some of which (like Fnr) are global regulators that control expression of respiratory chain components, globins, and enzymes that remove reactive dioxygen intermediates (ROI). The most important of these are superoxide dismutase (SOD) and catalases (cat). The respiratory chain is a source of ROI, but respiratory quinones (Q) are also important in limiting oxidative stress. Superoxide anion probably permeates into the cell from outside poorly, but respiratory generation of ROI in the periplasm may necessitate periplasmic SOD.

in higher ATP yields by oxidative phosphorylation than is use of NO_3^-/NO_2^- ($E^{\circ\prime}$ = +430 mV) or fumarate/succinate ($E^{\circ\prime}$ = +33 mV) (both of which may be used in bacterial anaerobic respiration) and pyruvate/lactate ($E^{\circ\prime}$ = −190 mV) (the last being an important reaction in fermentative energy conservation).

Oxygen, more correctly the dioxygen molecule, appeared in the atmosphere of the Earth about 2×10^9 years ago as a result of microbial photosynthetic activity. We imagine that organisms capitalized rapidly on the availability of such a useful oxidant as a thermodynamic sink, but the use of dioxygen in aerobic respiration is not without its difficulties. Its kinetic inertness requires activation by a metal centre which, in aerobic respiratory chains, generally comprises two transition metals, either a haem–haem couple or a haem–Cu couple (Poole, 2000). It has been suggested that these dioxygen-activating centres evolved from nitric oxide-reactive sites (Saraste and Castresana, 1994). Dioxygen is also only moderately soluble, an aqueous air-saturated solution containing about 200 μM dioxygen at 37°C. It is generally assumed that dioxygen, being a small uncharged molecule, will diffuse readily across biological membranes (Fig. 1) and that no significant dioxygen concentration gradient exists across respiring bacterial membranes (Unden *et al.*, 1995). Recent work suggests, however, that a metabolic advantage results from expression of microbial globins (Khosla and Bailey, 1989; Tsai *et al.*, 1996) which may act, as in higher organisms (Wittenberg and Wittenberg, 1990), to facilitate transport or storage of dioxygen for aerobic respiration. Even so, dioxygen-reducing oxidases have high affinities for the ligand, with K_m values typically in the sub-micromolar range.

Only the four-electron reduction of dioxygen to water is 'safe', since intermediate reduction products are toxic and reactive:

$$O_2 \longrightarrow O_2^- \longrightarrow O_2^{2-} \longrightarrow 2H_2O$$

Transfer of a single electron to dioxygen generates the superoxide radical anion (O_2^-), a highly reactive species that attacks many key biomolecules. The reactivity of dioxygen with metal ions, flavins and quinone-like molecules (autoxidation) will result *in vivo* in the liberation of superoxide anion. Superoxide production by neutrophils during the 'respiratory burst' (Henderson and Chappell, 1996) is a deliberate act of 'biological warfare', since the radical is used to attack engulfed bacteria. Superoxide production is also an unusual, apparently purposeful, feature of flavohaemoglobins and underlies the remarkable ability of these proteins to detoxify nitric oxide (Poole and Hughes, 2000). *In vivo*, superoxide is scavenged (Fig. 1) by superoxide dismutase (actually a family of metalloproteins with similar functions) that converts (dismutates) two molecules of superoxide to peroxide and water (Fridovich, 1995).

Transfer of a second electron to dioxygen or a single electron to superoxide

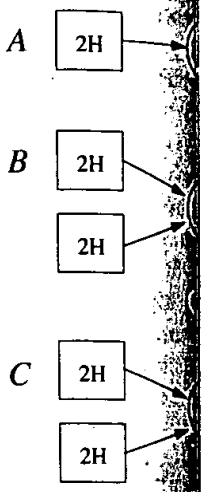
gives peroxide (O_2^{2-}), another reactive species that is scavenged *in vivo* by catalases and hydroperoxidases, a collective term for peroxide-consuming catalases and peroxidases (Loewen, 1996). The two-electron reduction of dioxygen to peroxide ($E^{\circ\prime} = 0.281$ V) is less energetically favourable than the complete reduction to water, but requires fewer electrons. Some oxidases, such as glucose oxidase, form peroxide as the major/sole product of dioxygen reduction but this is generally not the case when dioxygen is reduced in aerobic respiration. The high redox potential for H_2O_2 reduction to water (+1.349 V) is put to use by peroxidases; e.g. cytochrome *c* peroxidase. A further one-electron transfer to peroxide gives the hydroxyl radical ($OH^{\cdot}$) and the fourth electron yields water, by far the major product of aerobic respiratory dioxygen reduction.

The terminal oxidases of bacteria and eukaryotic mitochondria carry out some remarkable chemistry: typically, these enzymes bind, activate and reduce about 250 molecules of dioxygen per second, couple the energy released to proton translocation, yet release very little partially reduced dioxygen species. For a source of key, recent references to terminal oxidases, see Babcock (1999). Information on the major classes of bacterial oxidases can be found in reviews by Poole (1988, 1994, 2000), Saraste and Castresana (1994), Gennis and Stewart (1996), Poole and Hill (1997), and Delgado *et al.* (1998).

1.3. Architecture of Aerobic Respiratory Chains

In general, respiration achieves not only dioxygen consumption but also energy conservation by the generation of a protonmotive force (Δp) across an inherently proton-impermeable membrane. Respiratory chain components are usually associated with the membrane and are asymmetrically arranged across it. Such asymmetry allows electron transfer events to achieve net consumption of protons from the cytoplasmic compartment (or mitochondrial matrix) and net release of protons into the extracytoplasmic compartment (i.e. the periplasm) in Gram-negative bacteria (Nicholls and Ferguson, 1992). In the cytochrome *bd*-type quinol oxidase of *E. coli*, for example, the substrate (ubiquinol) is oxidized at the outer face of the membrane, where protons are released. Electrons then traverse the membrane via haems to a pocket presumably accessible from the cytoplasm. The electrons are used to reduce dioxygen to water, a step that requires protons taken from the inside, so that the net result is equivalent to proton translocation, although no protons in excess of the predicted redox stoichiometry have been 'pumped' (Gennis and Stewart, 1996).

Fig. 2 shows the key components or modules that comprise three generic classes of respiratory pathways of increasing complexity. Detailed description of the 'upstream' components in bacterial respiratory systems is outside the scope of this review, but details can be found in reviews and recent key papers



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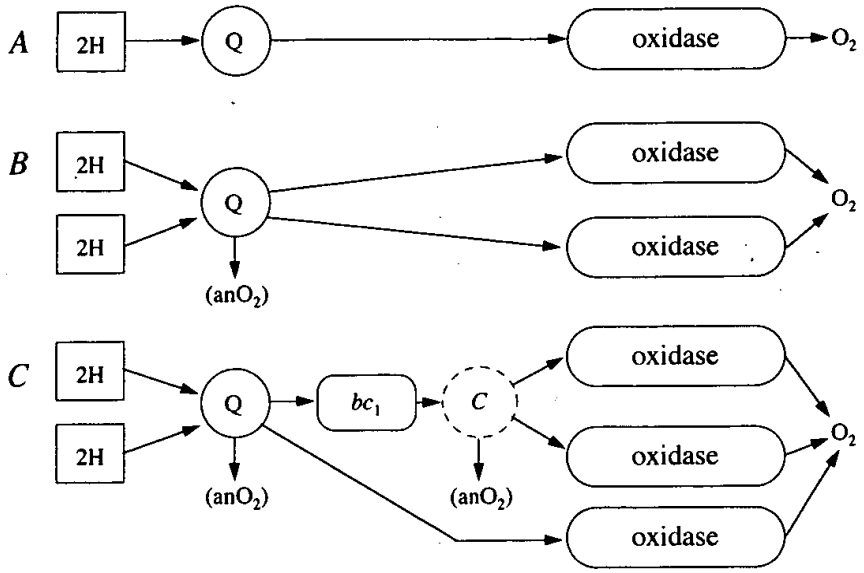


Figure 2 Basic plans for respiratory chains of increasing complexity. (A) A single dehydrogenase (2H) passes electrons to a quinone (Q) and a single oxidase reduces dioxygen. (B) Much more common are two or more oxidases and dehydrogenases, with alternative routes for anaerobic (anO_2) electron transfer to other oxidants. The respiratory chains of *E. coli* can be represented this way. (C) Multiple oxidases take electrons not only from Q but also cytochrome *c* via a route that may involve the cytochrome bc_1 complex.

on dehydrogenases (Gennis and Stewart, 1996), quinones (Gennis and Stewart, 1996; Søballe and Poole, 1999), *c*-type cytochromes (Iobbinivol *et al.*, 1994) and the cytochrome bc_1 complex (Trumpower and Gennis, 1994).

2. HISTORICAL PERSPECTIVE

The history of research on respiration is as long as the history of biochemistry, but several milestones are clearly visible (Keilin, 1970; Hempfling, 1979; Poole, 1988). By 1925, Keilin had conducted extensive experiments on the cytochromes present in many microorganisms and recommended a nomenclature based on the α-bands that is the basis of that used today. The diversity of cytochrome types was evident by the early 1930s. However, proof of the oxidase function of cytochromes *o*, *d* and a_1, and thus the idea that multiple oxidases coexist in a bacterial species, had to await the application by Chance, Smith and others of photochemical action spectroscopy. This elegant work

utilized the light sensitivity of the oxidase–CO adduct to demonstrate that its photolysis relieved the CO inhibition of respiration. Fortunately (in the absence of mutants and with fairly rudimentary information on how oxidase expression can be modulated by bacterial growth conditions), oxidases could be distinguished by their sensitivity to CO and light.

Decades of biochemical, physiological and genetic studies have culminated in today's landmarks – the availability of structural information on (a very limited number of) respiratory complexes and components and, second, the determination of entire genome sequences. Remarkable though these achievements are, they have so far contributed little to our understanding of the physiology of respiration and, particularly, the theme of this chapter. This will surely change.

3. RESPIRATORY CHAIN ORGANIZATION IN SELECTED BACTERIA

The most distinctive features of bacterial respiratory chains are their branched, flexible and adaptive nature. Simple, linear pathways involving a small number of dehydrogenases, a quinone and a terminal oxidase or reductase are uncommon; usually the respiratory pathway is branched at both ends and up to four or more terminal oxidases may be present. Fig. 2 shows standard plans of the composition and organization of the more common of these pathways.

Constructing such schemes requires integration of a number of experimental approaches, including determining the range of substrates that can be oxidized, the number and identity of cytochromes using spectroscopic analysis, chemical analysis of quinone types, functional dissection using mutants lacking one or more components, and deducing structural information by sequence analysis of genes. In a very few notable cases, crystallographic information has been obtained on protein structures. More recently, the availability of the entire sequences of bacterial genomes allows respiratory chain composition (but *not* function) to be predicted (but not determined) from observing what genes are present (and absent) and therefore what gene products may encode the respiratory pathways.

The presence of multiple oxidases is generally explained by the distinctive properties of each oxidase, which may differ from others in its affinity for dioxygen, its turnover number, or the stoichiometry of proton translocation, for example. Together with the ability of the respiratory chain to oxidize diverse respiratory substrates (e.g. NADH, succinate, D-lactate, etc.), this allows a bacterium to 'mix and match' different combinations of dehydrogenases and oxidases to achieve optimal substrate oxidation and energy conservation in a

variety of environmental conditions. Not surprisingly, then, the regulation of synthesis of these components is tightly regulated (see section 5).

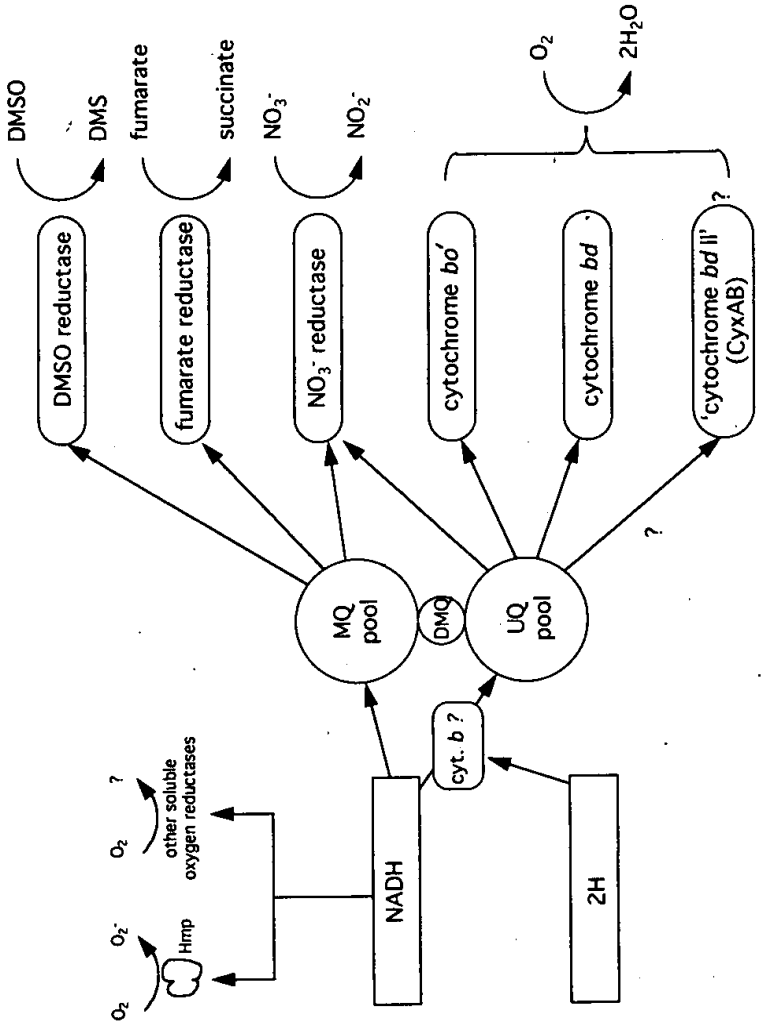
We have chosen to illustrate the organization of bacterial respiratory chains by describing each of a number of selected bacteria in turn, rather than describing the classes of oxidases and other respiratory chain components in turn. We do this in part because it seems that structurally similar components may exhibit quite distinct biochemical properties, and therefore physiological roles, according to the organism in which they exist. For example, cytochrome *bd* in *E. coli* (section 3.1) has a remarkably high affinity for dioxygen and allows this organism to respire aerobically at only trace levels of dioxygen. In *A. vinelandii*, however, (section 3.2) the same oxidase has an unremarkable affinity for dioxygen but supports such rapid rates of dioxygen consumption that, even under conditions of air saturation in the medium, dioxygen-labile nitrogenase in the cytoplasm still functions. Several bacteria whose respiratory metabolism is well documented have been omitted, generally because we have no further comments on, or explanations of, their multiple aerobic respiratory pathways. One such is *Paracoccus denitrificans* which has been extensively reviewed recently (Ferguson, 1998).

3.1. *Escherichia coli*

3.1.1. Overview

The respiratory chain of *E. coli* (Fig. 3) is a very well documented example of a complex branched arrangement of components which together result in the oxidation of a wide variety of substrates (e.g. NADH, succinate, malate, lactate, hydrogen, etc.; not detailed in Fig. 3). To a first approximation, electrons destined for transfer to dioxygen as terminal oxidant are transferred to ubiquinone and thence to one or both of two major oxidases, cytochromes *bo'* and *bd* (see later). Electrons destined for transfer to terminal oxidants other than dioxygen are transferred to menaquinone and thence to one or more major terminal reductases, only three of which are shown in Fig. 3. Note that nitrate reductase can accept electrons from either ubiquinone or menaquinone. As a result of these electron transfer reactions, protons are pumped or otherwise translocated from the cytoplasm outwards, thus generating a protonmotive force.

This model system exemplifies many of the most important characteristics of bacterial respiratory chains, namely (i) their branched nature at both 'dehydrogenase' and 'reductase' ends, (ii) the use of dioxygen or alternative electron acceptors, (iii) the presence of numerous types of cytochromes and quinones, (iv) 'cross-talk' between pathways optimizing the possibility of each reductant being paired with a wide choice of oxidants, and (v) concomitant proton translocation and energy transduction.



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The modularity of the respiratory apparatus makes possible the use of many different permutations of the 'low-potential' modules (i.e. the dehydrogenases, all of which are quinone reductases), quinones, and the 'high-potential' modules (i.e. the oxidases and reductases, all of which oxidize quinol). Apparent redundancy is evident at both ends of the respiratory pathways. For example, *E. coli* synthesizes two NADH dehydrogenases; although each transfers electrons from NADH to the quinone pool, the energetic consequences are different (Calhoun *et al.*, 1993). NDH-1 is a proton pump and results in translocation of protons with a stoichiometry of 2 H^+/e^-. NDH-II is not a pump and the ratio is 0 H^+/e^-. Likewise, the two major terminal dioxygen reductases are different in their coupling to proton translocation, with 2 H^+/e^- for cytochrome *bo'* and 1 H^+/e^- for cytochrome *bd* (Puustinen *et al.*, 1991). Other differences in these two oxidases are summarized in Table 1. It should be noted that early estimates of the dioxygen affinities for these oxidases, utilizing insensitive polarographic methods, have been superseded. The literature still contains statements to the effect that the K_m values for these oxidases are 10-fold different (e.g. Atlung and Brøndsted, 1994), but later measurements suggest that the K_m for dioxygen for cytochrome *bd* is about 100-fold lower than that for cytochrome *bo'* (references in Table 1). Other properties of cytochromes *bo'* and *bd* have been extensively reviewed (see Gennis and Stewart, 1996; Junemann, 1997).

3.1.2. *Roles in* E. coli *for Two Cytochrome* bd *Quinol Oxidases?*

A growing number of studies have revealed that cytochrome *bd* is required under conditions of environmental stress and may have crucial roles in cellular physiology other than acting as an oxidase. These roles are explored in more detail in section 4. Cytochrome *bd* is induced when *E. coli* is grown under

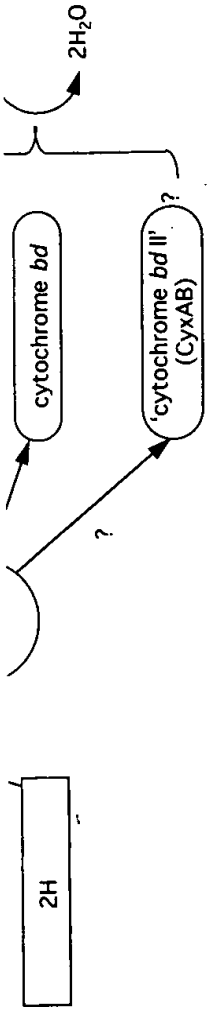

Figure 3 The respiratory chains of *E. coli*: a paradigm for the branched electron transport systems of facultatively aerobic bacteria. Dehydrogenases transfer electrons from numerous electron donors (only two are shown) to a pool of quinones. There is some evidence for the intermediary role of cytochrome(s) *b*. Generally, electrons from ubiquinone (UQ) are passed to terminal oxidases that use dioxygen or nitrate as terminal electron acceptors, whereas electrons from menaquinone (MQ) are taken to reductases that use anaerobic electron acceptors. Demethylmenaquinone (DMQ) is not important as a mediator of electron flux but is a biosynthetic intermediate with low activity in respiration. Dioxygen is reduced to water via two major oxidase complexes: cytochromes *bo'* and *bd*. A third oxidase ('*bd*-II') resembling the major cytochrome *bd* has been identified. Only two of the other many anaerobic reductases that reduce, respectively, dimethylsulfoxide (DMSO) to dimethylsulfide (DMS), and fumarate to succinate, are shown. NADH can also be oxidized by at least two soluble pathways of electron transfer to dioxygen, one of which involves the flavohaemoglobin, Hmp. See text for details.

Table 1 Comparison of the functional properties of oxidases in *Escherichia coli*.

	Cytochrome *bo'*	Cytochrome *bd* ('*bd*-I')	Cytochrome *bd* ('*bd*-II')	Flavohaemoglobin
Genes required for synthesis	*cyoABCDE*[1]	*cydAB*, *cydDC*[1]	*cbdAB* (also *cyxAB*, *appCB*)[1,2] *cydDC*?	*hmp*[3]
Subunit or protein mass (kDa)	35, 75, 23, 12, 32*,[1]	58, 42*,[1] (CydA, CydB) 48, 26.5*,[2] (CydA, CydB)	+ ?? 43, 27*,[2]	44**,[3]
Redox prosthetic groups	cytochromes b_{562} and o', Cu_B[1]	cytochromes b_{558}, b_{595}, d[1]	cytochromes b_{558}, b_{595}, d (?)[2]	cytochrome *b*, FAD[4]
Product of dioxygen reduction	H_2O[1]	H_2O[1]	?	O_2^-, O_2^{2-}, H_2O[5,6]
K_m for dioxygen (μM)	0.016–0.35[7]	0.003–0.008[8]	?	2.6[9]
Reductant	ubiquinol[1]	ubiquinol[1]	ubiquinol[2]	NAD(P)H[4,6,9]
H^+ pump, H^+ translocation	yes, $H^+/e^- = 2$[1]	no, $H^+/e^- = 1$[1]	?	not applicable (soluble)
Cyanide conc. (mM) giving approx. 50% inhibition	0.01[10]	2[10]	0.5 (67% inhibition)[2]	0.03 (50% fractional saturation)[11]

* approximate, based on DNA sequence analysis
*apparent on SDS-PAGE
** based on DNA sequence analysis and gel filtration

References: 1. Gennis and Stewart (1996); 2. Sturr *et al.* (1996); 3. Vasudevan *et al.* (1991); 4. Ioannidis *et al.* (1992); 5. Membrillo-Hernández *et al.* (1996); 6. C. E. Mills, B. Søballe, R. K. Poole, in preparation; 7. D'mello *et al.* (1995); 8. D'mello *et al.* (1996a); 9. Poole *et al.* (1996); 10. Kita *et al.* (1984); 11. N. Ioannidis and R. K. Poole, in preparation.

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unfavourable growth conditions (Avetisyan *et al.*, 1991; Bogachev *et al.*, 1993, 1995). Mutants that cannot synthesize cytochrome *bd* are sensitive to H_2O_2 and are temperature-sensitive. Both of these observations are consistent with *cydAB* being heat-shock genes (Wall *et al.*, 1992). Unlike the classical heat-shock regulon (*dnaK*, *groE*, and *lon*) that is regulated by σ^{32}, *cydAB* expression is not. The heat-shock induction of *cydAB* is under Arc regulation. Mutants defective in cytochrome *bd* are also sensitive to a self-produced extracellular factor that inhibits their growth (Macinga and Rather, 1996; Cook *et al.*, 1998; Section 4.5). Recently two new proteins of unknown function have been shown to be encoded by the *cydAB* operon (Muller and Webster, 1997); might these play a key role in the unexpectedly complex phenotype of Cyd^- mutants?

The genes known as *cyxAB* (Sturr *et al.*, 1996), and also as *appCB* and *cbdAB*, are part of an operon at 22 min on the *E. coli* chromosome (*cyxAB appA*). *appA* encodes an acid phosphatase but, surprisingly, *cyxAB* show clear homology with the *cydAB* operon that encodes the two subunits of cytochrome *bd* quinol oxidase (Dassa *et al.*, 1991). The *cyxAB* genes are cotranscribed with *appA* from a potentially strong promoter immediately upstream of *cyxA*, which is activated by anaerobic growth conditions, phosphate starvation and entry into the stationary phase (Atlung and Brøndsted, 1994), as well as high osmolarity (Atlung *et al.*, 1997). AppY up-regulates the *cyx* promoter and an *appY* mutation eliminates or markedly reduces response to the above stresses. Nevertheless, the *appY* mutant is unaffected in stationary-phase survival, or growth and yield in aerobic or anaerobic culture. In turn, *appY* is regulated by RpoS, Fnr and ArcA, suggesting complex regulation in response to diverse environmental stresses. Recently, expression of *appY* has been shown to be regulated also in response to dioxygen via a novel two-component system DpiB/DpiA (for references, see Sawers, 1999). It is thought that this system functions to prevent expression of *appY* when dioxygen is available, and it follows that expression of this cytochrome *bd* is normally shut off aerobically.

Clues to a function for the *cyxAB*-encoded proteins, which perhaps should be called cytochrome *bd*-II, come from the fact that *cyxAB*$^+$ *cyo cydAB* strains do form microcolonies on media provided only with non-fermentable substrates, indicating some respiratory function (Gennis and Stewart, 1996). The triple mutant (*cyo cydAB cyxA*) is more dioxygen-sensitive than the *cyxA*$^+$ double mutant (Dassa *et al.*, 1991). Importantly, Sturr *et al.* (1996) have demonstrated that the *cbdAB* genes can encode an oxidase. Surprisingly, a recombinant plasmid containing DNA from alkaliphilic *Bacillus firmus* OF4 was able to complement an *E. coli* mutant defective in *cyo* and *cydAB* for growth on succinate. Membrane vesicles from the transformant exhibited cytochrome *bd* spectral signals. The putative oxidase was partially purified and an internal peptide was shown to correspond with a sequence predicted for the *appC* gene product. This oxidase is immunologically distinct from the *cydAB*-encoded oxidase yet functions *in vitro* as a quinol and TMPD oxidase.

Compared with the *cydAB*-encoded oxidase, this activity is relatively sensitive to cyanide, and there is no evidence for a role in Na^+-motive or Na^+-dependent respiration (Sturr *et al.*, 1996).

3.1.3. Soluble Oxidases

Membrane-free fractions prepared from disrupted *E. coli* cells have readily measurable dioxygen consumption activities but very little is known of their nature or role(s). Recent work has shown that one component is the flavohaemoglobin Hmp, which reduces dioxygen to give mostly superoxide (see references in Table 1). Other components probably include various 'diaphorases' that may function in maintenance of reduced states of certain proteins and may be involved, for example, in reduction of dyes such as methyl viologen (paraquat) (Liochev *et al.*, 1994). Another possible contributor might be reductases analogous to diaphorases that maintain quinones in reduced forms (see section 3.1.4). Although unlikely to be important in energy conservation, these poorly understood oxidase activities might have essential roles in stress responses.

3.1.4. Additional Roles for Quinones?

E. coli synthesizes three types of quinones: a benzoquinone (UQ-8, having eight prenyl units in the side-chain), and two naphthoquinones, menaquinone (MK) and demethylmenaquinone (DMK) (Søballe and Poole, 1999). The octaprenyl side-chain renders them soluble in the cytoplasmic membrane. Only during aerobic respiration on non-fermentable substrates, such as succinate, is UQ absolutely required. As we have seen, aerobic respiration carries the risk of generation of partly reduced products of dioxygen metabolism, in particular superoxide and peroxide. Although terminal oxidases achieve dioxygen reduction yet minimize the formation of these toxic species, other segments of the respiratory chain can generate these forms. In particular, the ability of quinones to transfer electron pairs in discrete one-electron steps means that O_2 can inadvertently accept electrons to generate superoxide anion ($O_2^{\cdot-}$), which directly or via peroxide can damage many biomolecules. Indeed, some naturally produced quinones (e.g. plumbagin and juglone) exert antimicrobial activity via 'redox cycling' – the reduction of dioxygen to $O_2^{\cdot-}$ followed by quinone re-reduction. Consequently, quinones in respiratory chains may have pro-oxidant activities (Beyer *et al.*, 1996).

Recent attempts to determine the sites in respiratory chains where $O_2^{\cdot-}$ is generated have used inhibitors of electron transfer and certain dehydrogenase and oxidase mutants (González-Flecha and Demple, 1995). This work showed that the *E. coli* respiratory chain is responsible for 90% of cellular H_2O_2

production (assumed to result from primary $O_2^{\cdot-}$ generation) and that production rates 'were linearly related to the number of active respiratory chains'. Imlay (1995) suggests that quinones do not contribute to $O_2^{\cdot-}$ generation but the experiments used a leaky *ubiA* allele to achieve a strain lacking ubiquinone and did not take into account $O_2^{\cdot-}$ from residual electron transfer to the oxidases. Using a knockout *ubiCA* mutant, Søballe and Poole (2000) have shown that large amounts of superoxide and peroxide accumulate in membranes from a Ubi^- strain, in contrast to wild-type membranes which possess superoxide-scavenging ubiquinol. Expression of the *katG* gene, encoding the catalase hydroperoxidase I, as well as catalase enzyme activity, are also increased two-fold in the *ubiCA* mutant, which is hypersensitive to oxidative stress mediated by $CuSO_4$ or H_2O_2. These observations support the participation of reduced ubiquinone as an antioxidant in *E. coli*.

Ubiquinone acts as an antioxidant in the fully reduced quinol or hydroquinone form to scavenge free radicals. How is this state maintained? In higher organisms, the hydroquinone is the dominant form of UQ in diverse, even non-respiring, membranes and is maintained by the activity of quinone reductases (the best known being hepatocyte DT-diaphorase) that catalyse *two*-electron reduction of quinone substrates and thus protect against cytotoxic and carcinogenic effects (Beyer *et al.*, 1996). It is not known whether bacteria possess such a mechanism, but *E. coli* does contain a menadione-inducible NADH-Q reductase and a quinone oxidoreductase (Qor) (Thorn *et al.*, 1995). The substrates for these enzymes may be exogenous water-soluble quinones or the membrane-bound 'respiratory' quinones.

3.2. *Azotobacter vinelandii*

In the free-living diazotrophic bacterium *Azotobacter vinelandii*, electrons from ubiquinone are transferred to at least two (and perhaps three) oxidases, each with distinct structural, kinetic and functional properties. This system has been recently reviewed by Poole and Hill (1997). The best studied oxidase is cytochrome *bd*, because of the genetic evidence for its essential role in aerotolerant nitrogen fixation (Kelly *et al.*, 1990). This is considered further in section 4.1. The identity and role of the one or two alternative oxidases is far less clear. A fragment of a gene encoding an oxidase in the haem–Cu family has been cloned and mutated, resulting in the conclusion that this oxidase is not essential for nitrogen fixation. However, evidence for an oxidase of the *fixN* or *ccoN* type (*cbb'*) has also been presented; it is not clear whether there are one or two oxidases in addition to cytochrome *bd*, but determinations of K_m values suggest two, both of higher affinity for O_2 than cytochrome *bd*.

3.3. *Klebsiella* Species

Klebsiella pneumoniae is a facultative anaerobe that fixes N_2 anaerobically by fermentative metabolism; no N_2 fixation occurs under aerobic growth conditions (Hill *et al.*, 1990). However, microaerobic growth conditions can benefit diazotrophy when measured by the amount of N_2 fixed per unit of carbon and energy source consumed (Hill *et al.*, 1990). Under these conditions the maximum dissolved O_2 concentration tolerated is about 30 nM. In contrast, the free-living obligate aerobe *Azotobacter* (section 3.2) can fix N_2 over a wide range of O_2 concentrations.

K. pneumoniae synthesizes *b*- and *d*-type cytochromes over a wide range of growth conditions and has a branched respiratory chain terminating in two oxidases of the cytochrome *bo′* and *bd*-types (Smith *et al.*, 1990). Cytochrome *bo′* predominates under high aeration and cytochrome *bd* is dominant under microaerobic or anaerobic growth conditions. Like the cytochrome *bd* of *E. coli*, the affinity for dioxygen is extraordinarily high (K_m = 20 nM; Smith *et al.*, 1990). Although cytochrome *bd* appears to have many physiological roles that are not well understood (see section 4), in *Klebsiella* one important role is to scavenge dioxygen that would otherwise inactivate the dioxygen-sensitive nitrogenase. Indeed, cytochrome *bd* is the only oxidase expressed under nitrogen-fixing conditions (Smith *et al.* 1990). Juty *et al.* (1997) have demonstrated the role of cytochrome *bd* in microaerobic nitrogen fixation using mutants defective in cytochrome *bd*, which were severely impaired in their ability to fix nitrogen in the presence of dioxygen. Furthermore, a role in conservation of energy under microaerobic growth conditions was demonstrated. The authors proposed that formate oxidation by formate dehydrogenase-O is able to provide electrons for an electron transport chain terminating in cytochrome *bd*, which would remove inhibitory dioxygen and supply ATP for nitrogenase activity.

In addition to the two major terminal respiratory oxidases in *K. pneumoniae*, cytochromes *d* and *o*, Chena and Liu (1999) have identified a non-haem terminal oxidase in *Klebsiella oxytoca* growing in medium containing KCN. The expression of *b*-type cytochromes and cytochrome *d* decreased by 50%, while cytochrome *bo′* increased by 70%, but spectral analysis could detect no new cytochromes that were induced under KCN stress. Two terminal oxidases were observed in *K. oxytoca* during growth in medium containing KCN based on K_i values for the inhibitor. From this study, the authors propose the presence of a non-haem type of terminal oxidase, possibly an iron-sulfoprotein, which accounts for KCN resistance in *K. oxytoca*. Caution should be exercised, however: first, spectrally undetectable levels of oxidase might support substantial oxidase activities (see calculations in Poole and Hill, 1997); second, oxidases, even when identified at a molecular level, might not display distinctive spectroscopic properties (see section 3.5).

3.4. Rhizobia

During free-living growth, bacteria from the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively known as rhizobia) have branched electron transport chains that are terminated by cytochromes aa_3 and cytochrome *o'* and/or *d* (O'Brian and Maier, 1989; Bott *et al.*, 1990; Kitts and Ludwig, 1994; Delgado *et al.*, 1998). These microorganisms respire using both quinol-dependent and cytochrome *c*-dependent terminal oxidases, and the cytochrome composition of the electron transport chain of different *Rhizobium* species has been thoroughly reviewed recently (Delgado *et al.*, 1998). As is the case for respiratory oxidases in other bacterial genera, dioxygen plays an important role in the regulation of expression of these oxidases. A key feature of the respiratory metabolism of rhizobia and other nitrogen-fixing bacteria is the use of a specialized oxidase to decrease the partial pressure of O_2 and permit N_2 fixation. In contrast to *Azotobacter* and *Klebsiella*, which use cytochrome *bd* to keep the partial pressure of dioxygen low, *Rhizobium* and *Bradyrhizobium* species use an alternative terminal oxidase known as cytochrome 'cbb_3'* (Preisig *et al.*, 1993, 1996a,b), encoded by the genes *fixNOQP* (Preisig *et al.*, 1996b). Typically, it accounts for 85% of the total cytochrome *c* oxidase activity in bacteroid membranes and supports microaerobic respiration in endosymbiotic bacteroids.

The dioxygen affinities of some oxidases in these bacteria are appropriately high. For example, *Rhizobium leguminosarum* bacteroids possess a respiratory chain terminating with a very high-affinity oxidase, the K_m for dioxygen – measured using a suitably sensitive oxyglobin deoxygenation method – being 45 nM (Haaker *et al.*, 1996). A *fixNOQP* operon, presumed to encode a high-affinity bacteroid-specific cytochrome *cbb'*-type oxidase, is present on the symbiosis plasmid and is highly induced in microaerobic conditions (Gutierrez *et al.*, 1997). The K_m for dioxygen of the *cbb'*-type oxidase in membranes of *Bradyrhizobium japonicum* is 7 nM, the lowest reported for a haem–Cu oxidase (Preisig *et al.*, 1996a,b).

Bradyrhizobium japonicum, a symbiotic nitrogen-fixing bacterium, has a complex respiratory electron-transport chain, capable of functioning throughout a wide range of dioxygen tensions. Spectral, inhibitor, and O_2-consumption studies on membranes from free-living and bacteroid forms of *B. japonicum* have revealed the existence of a number of terminal oxidases, and four terminal oxidase gene clusters within the haem–Cu cytochrome family have been cloned (Bott *et al.*, 1992; Preisig *et al.*, 1993, 1996a,b; Surpin *et al.*, 1996; Zufferey *et al.*, 1996). At present, it is unknown what roles these individual

* Note that this name is not in accord with recommended biochemical nomenclature for haemproteins. Cytochrome *cbb'* might be a better name (Poole and Chance, 1995), where the 'prime' indicates the putative ligand-binding haem, as does the subscript '3' in 'cytochrome aa_3'. In this review, this nomenclature will be used to describe cytchromes *bo'* (bo_3), *cao'* (cao_3), and *bb'* (bb_3).

oxidases play. Under aerobic growth conditions the respiratory chain of *B. japonicum* terminates with an aa_3-type (CoxBA) cytochrome *c* oxidase. Mutations in this oxidase do not disturb bacteroid development and symbiotic nitrogen fixation (Bott *et al.*, 1990). Likewise, a mutation in the cytochrome *c* oxidase, CoxMNOP, does not have any effect on symbiosis and nitrogen fixation (Bott *et al.*, 1991). A fourth terminal oxidase has been described in *B. japonicum* encoded by the *coxWXYZ* operon (Surpin *et al.*, 1994, 1996). This oxidase is a cytochrome *bb'*-type ubiquinol oxidase (Surpin *et al.*, 1996) and is preferentially expressed under microaerobic growth conditions. Studies with *coxX* mutants have shown that both the viable cell number recovered from crushed root nodules and the rate of nitrogenase activity was 20–40% lower than for the wild type or $CoxN^-$ strain, suggesting an important role for the CoxWXYZ terminal oxidase in symbiosis (Surpin and Maier, 1999). Furthermore, Surpin and Maier (1998) have demonstrated that a mutant defective in *coxWXYAZ* and *coxMNOP* was severely deficient in H_2-dependent chemolithotrophic growth. This growth condition requires prolonged incubation in an atmosphere of H_2, CO_2, and a low (1% or less) partial pressure of dioxygen.

The *cbb'*-type of cytochrome *c* oxidase has also been reported in the non-endosymbiotic photosynthetic bacterium *R. sphaeroides* when grown under microaerobic conditions (Toledo-Cuevas *et al.*, 1998), in *Agrobacterium tumefaciens* (Schluter *et al.*, 1995) and in *Rhodobacter capsulatus* (Thony-Meyer *et al.*, 1994).

Overall, the complement of respiratory oxidases present in rhizobial species enables them to cope with a wide range of dioxygen concentrations. The cytochrome *cbb'* oxidase encoded by *fixNOQP* functions at nanomolar levels of free dioxygen in mature bacteroids, whereas the CoxWXYZ oxidase functions at micromolar levels of dioxygen in the early stages of mature bacteroid or nodule development. CoxMNOP apparently does not function in, or significantly alter, the symbiosis (Surpin *et al.*, 1994; Surpin and Maier 1999). The extreme case of *Azorhizobium caulinodans*, claimed to have at least five oxidases, is considered in section 6.

3.5. *Pseudomonas aeruginosa*

Pseudomonas is an opportunistic pathogen with a branched aerobic respiratory chain that terminates in *b*- and *c*-type cytochromes (Matsushita *et al.*, 1980a,b, 1982a,b, 1983). There are at least two terminal oxidases: cytochrome *co*, a cytochrome *c* oxidase possibly related to the *cbb'*-type oxidase (haem–Cu) family, and a novel type of cytochrome *c* oxidase that is a baa_3-type oxidase (Fujiwara *et al.*, 1992). Okamoto *et al.* (1995) have shown that the reactivity of these two cytochrome *c* oxidases differs. Cytochrome *co* reacts more rapidly

with membrane-bound cytochrome c_{551}, whereas cytochrome baa_3 reacts with membrane-bound cytochrome c_{555}. Intriguingly, mutants that lack *c*-type cytochromes have a functional cyanide-insensitive oxidase (Ray and Williams, 1996) encoded by the *cioAB* genes that are clearly related to genes encoding cytochrome *bd* in *E. coli* (Cunningham and Williams, 1995; Cunningham *et al.*, 1997). Mutants defective in *cioAB* exhibit no net loss of spectral signals and no signals indicative of cytochrome *bd* under conditions of high or low aeration (Cunningham *et al.*, 1997). The nature of the redox-active centres that endow the deduced CioA and CioB subunits with oxidase activity remains unknown.

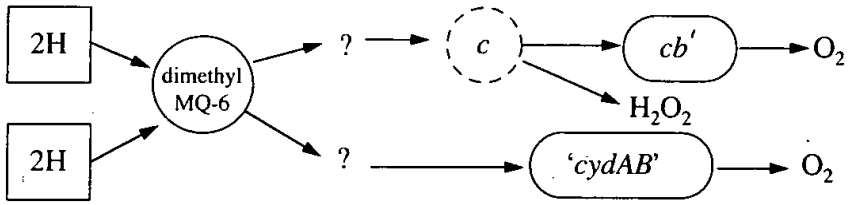
A role for the CioAB oxidase in the growth of *Pseudomonas* has been proposed. The acquisition of the ability to produce HCN is preceded by dioxygen reduction to growth-limiting conditions. *Pseudomonas* produces cyanide under low dioxygen tensions that inhibits haem–Cu oxidases, and so the function of CioAB may be to allow aerobic respiration under cyanogenic growth conditions (Castric, 1983).

3.6. *Campylobacter* Species

Campylobacter jejuni and *Campylobacter coli* are probably the major bacterial causes of food-associated human disease in the developed world. Although rarely fatal, the gastroenteritis caused is debilitating and very unpleasant. A few cases result in chronic illness such as reactive arthritis and Guillain–Barré syndrome. From 1981 to 1997, diagnosed cases of campylobacter-related problems rose from 12 to 50 thousand in England and Wales with substantial year-on-year increases. Campylobacteriosis is thus an economically significant food-borne disease. *C. jejuni* and *C. coli*, the species most important for human disease, are Gram-negative, spiral-shaped bacteria, which are commensal microflora of the gastrointestinal tracts of poultry and other birds, cattle, pigs and other animals. Campylobacters penetrate the intestinal epithelium and mucosa and proliferate; they may also produce a cholera-like enterotoxin. However, the mechanisms of pathogenicity are poorly understood.

Campylobacters have the unique property – for a food-borne pathogen – of being microaerophilic: they require at least 3% dioxygen for growth but 5–7% dioxygen is optimal. However, they must be able to survive in air during transmission and persist in foods. The availability of dioxygen is likely to cause profound changes in the physiology of these pathogens, which may relate to their ability to survive in food and cause infection. The metabolism of campylobacters is strictly respiratory, but dioxygen utilization is very poorly understood. Early studies revealed *c*- and *b*-type cytochromes, including a CO-reactive cytochrome *c′* considered to be a candidate for an oxidase (Harvey and Lascelles, 1980; Hoffman and Goodman, 1982). Membranes exhibited

Campylobacter sp.



Helicobacter pylori

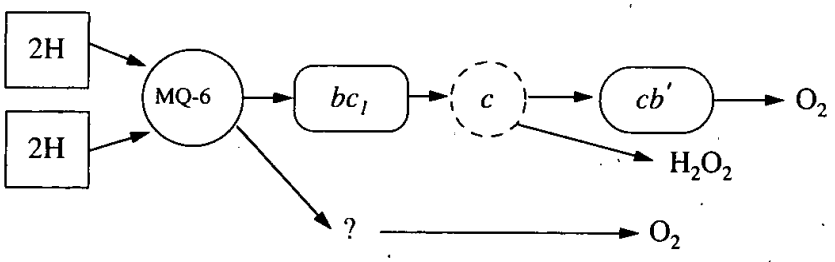


Figure 4 Possible respiratory electron transport chains in *Campylobacter* sp. (top) and *Helicobacter pylori*. In both bacteria, several dehydrogenases feed electrons to menaquinone (MQ) or a MQ derivative. Both bacteria are rich in *c*-type cytochromes, which are in part involved in oxidases of the *cb'*-type. Cytochrome *c* appears to be a branch point for electron transfer to peroxide via cytochrome *c* peroxidase. Genome sequence and some spectroscopic data suggest an additional oxidase related to cytochrome *bd* in both bacteria. See text for details.

particularly high oxidase activities with formate or hydrogen as reductants, and reactivity with viologen dyes indicated that both formate dehydrogenase and hydrogenase were located at the outer face of the cytoplasmic membrane. Two oxidase activities, unassigned to particular oxidase complexes, were suggested by the biphasic pattern of respiratory inhibition by cyanide (Hoffman and Goodman, 1982). Interestingly, respiration was relatively insensitive to inhibition by CO. The *b*-type cytochromes were not characterized, but their involvement with cytochrome *c'* in an oxidase complex was suggested; such an oxidase would presumably oxidize directly or indirectly the novel dimethyl menaquinone-6 identified by Collins *et al.* (1984) as the only respiratory quinone.

The cytochrome components were analysed in more detail by Lascelles and Calder (1985). Membranes contained *c*- and *b*-type cytochromes, but CO difference spectroscopy revealed a predominant peak at about 410 nm, suggesting that the *c*-type cytochrome was the major CO-reactive haemprotein.

Reaction of CO with succinate-reduced membranes revealed, in addition to the CO-reactive cytochrome *c*, an absorbance at 422 nm (in the CO-ligated state) that might suggest a cytochrome *o*′-like oxidase (Lascelles and Calder, 1985). However, the complete genomic sequence (to be published) reveals no *cyo*-like genes encoding a cytochrome *o*′, but instead genes that are expected to encode an oxidase of the *fixN* (*cbb*′) type. There are no spectral signals attributable to cytochromes aa_3 or *bd*. The view that emerges from these very incomplete studies is intriguing and summarized in Fig. 4.

Of special importance is understanding the basis of microaerophily. In neither *Campylobacter* nor *Helicobacter* (section 3.7) is this understood. Dioxygen sensitivity of key enzymes is a possibility, as discussed by Kelly (1998) in the context of pyruvate oxidoreductase. Other untested possibilities are that (i) respiratory electron transfer is excessively uncoupled from energy transduction, leading to unnecessary and excessive dioxygen radical production; (ii) oxidase activity is inhibited by excess dioxygen, as seems to be the case for *E. coli* cytochrome *bd* (section 5); or (iii) an oxidase with low dioxygen affinity (operative at high dioxygen) produces oxygen radicals, whilst a high-affinity oxidase (operative at low dioxygen) produces fewer oxygen radicals.

Goodhew *et al.* (1988) suggest that aerobic metabolism produces substantial hydrogen peroxide levels which, on the one hand, are inhibitory yet, on the other, may allow energy conservation by accepting electrons from the respiratory chain by peroxidase activity. Lack of superoxide dismutase or catalase to remove deleterious dioxygen reduction products is unlikely to explain the dioxygen sensitivity. Indeed, inactivation of *sodB*, encoding an Fe-SOD, did not affect the ability of cells to grow aerobically, but severely compromised the ability of cells to survive under aerobic conditions in laboratory media and in food. SOD-deficient mutants also exhibited decreased colonization potential in an experimental oral infection of one-day-old chicks, indicating that SOD is required for optimal survival within the chicken gut (Purdy *et al.*, 1999). Although this work has provided some insight into the physiology of dioxygen tolerance in campylobacters, the molecular events associated with the sensing of dioxygen concentration and those involved in the cellular responses to dioxygen and its reduction products remain uncharacterized.

3.7. *Helicobacter pylori*

The microaerophilic bacterium *Helicobacter pylori* colonizes the mucous layer of the human gastric epithelium and is now known to be the aetiologic agent of chronic active gastritis and duodenal ulceration. It is thus one of the most common pathogens worldwide, infecting, for example, about 30–50% of the

population of Western Europe. The microaerophilic nature of the bacterium is of special interest for the purposes of this review: how is energy conservation accomplished and what aspects of the organism's physiology prevent it from growing under aerobic conditions? The complete genomic sequence of a pathogenic strain has been determined recently and analysed (see Kelly, 1998, for references) in the context of the organism's physiology and metabolism. With respect to respiration and energetics, this exercise has lent support to some earlier studies but questioned others.

Collectively, the biochemical and genomic approaches suggest a surprisingly simple organization for the respiratory chain (Fig. 4), reviewed by Kelly (1998). NADH, NADPH, fumarate, D-lactate and succinate are rapidly oxidized by isolated membranes; NADPH is oxidized much faster than NADH (Chang *et al.*, 1995). Menaquinone-6 is the major isoprenoid quinone and *b*- and *c*-type cytochromes are evident spectroscopically, but not cytochromes *a* or *d* (Marcelli *et al.*, 1996). These results are consistent with analysis of the genome sequence (see Kelly, 1998), which suggests that electrons fed into the quinone from diverse dehydrogenases are transferred to dioxygen via a cytochrome bc_1 complex and a soluble cytochrome *c* to a single oxidase. The nature of the oxidase is of special interest in view of the possibility that the dioxygen kinetics of the oxidase might underlie the microaerobic growth physiology. There is both genetic and biochemical evidence for an oxidase of the cytochrome *cbb'*-type, encoded by *fixNOQP*. In symbiotic rhizobia (section 3.4), such an oxidase has an exceptionally high affinity for dioxygen (K_m about 7 nM), but the measured K_m in *H. pylori* is only 0.4 μM (Nagata *et al.*, 1996). The high sensitivity to cyanide of this oxidase appears inconsistent with the finding that lactate respiration is relatively cyanide-resistant (for references, see Kelly, 1998), suggesting the presence of a branched respiratory chain and an additional cyanide-insensitive oxidase, such as cytochrome *bd*. Indeed, spectral signatures of such an oxidase have been reported in membranes from a clinical isolate of *H. pylori*, but the *cydAB* genes that would be expected to encode such an oxidase are absent from the genome of strain 26695 (for references, see Kelly, 1998). Fumarate appears to be a possible acceptor of electrons from the quinone pool (Chen *et al.*, 1999). Reconciling and integrating results from several approaches will be an important target for future work.

3.8. *Bacillus subtilis*

During aerobic respiration, *Bacillus subtilis* utilizes a branched respiratory system comprising various cytochromes of the *a*-, *b*-, *c*-, *d*- and *o*-types (von Wachenfeldt and Hederstedt; 1990, 1992; Schiott *et al.*, 1997; Winstedt *et al.*, 1998). The cytochrome composition of *B. subtilis* membranes isolated from

vegetative cells varies depending on the strain, the growth conditions and growth stage. Eleven different cytochromes or cytochrome-containing enzyme complexes have so far been identified in *B. subtilis* (von Wachenfeldt and Hederstedt, 1992).

At present, there is biochemical and genetic evidence for three types of terminal oxidases, a cytochrome caa_3 encoded by the *ctaABCDEF* operon, a cytochrome aa_3 encoded by *qoxABCD*, and a cytochrome *bd*-type oxidase encoded by the *cydABCD* operon (Winstedt *et al.*, 1998). The first of these is believed to function as a cytochrome *c* oxidase, whereas the latter two use menaquinol as a substrate (von Wachenfeldt and Hederstedt, 1992; Winstedt *et al.*, 1998). Both *a*-type oxidases are members of the well-characterized haem–Cu superfamily of terminal oxidases. Mutants defective in cytochrome caa_3 showed similar growth properties and colony formation to a wild-type strain on rich and minimal medium (van der Oost *et al.*, 1991). Comparatively, a cytochrome aa_3-deficient mutant had a small colony phenotype and grew more slowly than the wild-type strain. Mutants defective in both oxidases show an increase in the expression of cytochrome *d* (van der Oost *et al.*, 1991). In terms of cytochrome expression very little is known, but synthesis appears to be dependent on the growth medium being used. For example, cytochrome aa_3 dominates in rapidly growing cells (glucose-containing medium), whereas cytochrome caa_3 is predominantly expressed in media allowing slow growth (e.g. minimal medium containing succinate). Based on the small colony phenotype and slow growth rate of an aa_3 mutant, cytochrome aa_3 appears to be the most important terminal oxidase during vegetative growth. The reason is unknown.

Membranes from wild-type cells grown with glucose or from strains lacking haem A synthesize a *d*-type cytochrome (van der Oost *et al.*, 1991). The optical spectrum of cytochrome *d* is similar to that of the *E. coli* cytochrome *bd* complex. In contrast to *E. coli* Cyd$^-$ mutants, *cyd* mutants of *B. subtilis* show no apparent effect on growth of cells in broth or defined medium (Winstedt *et al.*, 1998). The regulation of cytochrome *bd* in *B. subtilis* is at the level of *cydABCD* transcription. In *E. coli*, the *cydDC* genes are found in an operon separate from *cydAB* (Poole *et al.*, 1993) and are not coordinately regulated (Cook *et al.*, 1997). In *B. subtilis*, *cydABCD* form an operon that is expressed as a single transcript (Winstedt *et al.*, 1998). When cells are grown with high aeration, cytochrome *bd* is repressed. When the dioxygen tension is lowered, expression of *cyd* is induced and reaches its maximum during the transition from exponential to stationary growth phase. At present, it is not known what regulatory proteins control this expression in *B. subtilis* or what physiological advantage accrues from this oxidase. In addition, there is spectroscopic evidence for at least one cytochrome *o*-type oxidase in *B. subtilis* (James *et al.*, 1989).

3.9. *Zymomonas mobilis*

The Gram-negative bacterium *Zymomonas mobilis* is an aerotolerant, ethanol-producing anaerobe. Although notable for its fermentative metabolism and the production of ethanol, *Z. mobilis* possesses a branched respiratory chain (Kalnenieks *et al.*, 1998) with oxidative phosphorylation capacity (Kalnenieks *et al.*, 1993) that endows the organism with the ability to respire at rates comparable to those of *A. vinelandii* (Pankova *et al.*, 1988; Kalnenieks *et al.*, 1995; U. Kalnenieks *et al.*, in preparation). Growth conditions appear to affect the nature of the respiratory chain. Under aerobic conditions, an increase of the cytochrome α-peak in the reduced *minus* oxidized difference spectra was reported (Kalnenieks *et al.*, 1996). Moreover, in anaerobically grown cells, oxidative phosphorylation activity is linked solely to 'site I' (NADH dehydrogenase), while in aerobically grown cells it shifts to the cytochrome region of the respiratory chain (Kalnenieks *et al.*, 1995, 1996), and the energy non-generating NADH dehydrogenase of type II prevails (Kalnenieks *et al.*, 1996). More recent analysis of the respiratory chain reveals, in addition to ubiquinone-10, the presence of cytochrome *bd*, a cytochrome *o'*-like CO-binding haemprotein, and probably a cytochrome *a*-type oxidase (Kalnenieks *et al.*, 1998).

The physiological role of respiration in *Z. mobilis* is still obscure. It clearly does not serve as an energy source for aerobic biomass growth in the way respiration does in most facultatively anaerobic and aerobic bacteria (Pankova *et al.*, 1985), as judged from the low aerobic biomass yields; the highest reported biomass yield values for *Z. mobilis* are about 20 g of dry biomass per mole of glucose in aerobic chemostat cultures. The fermentative catabolism of *Z. mobilis* is well balanced, yielding 2 mol of ethanol per mole of catabolized glucose via the Entner–Doudoroff pathway and appears not to supply additional reducing equivalents to the respiratory chain. In aerobic culture, the respiratory chain competes for NAD(P)H with the alcohol dehydrogenase reaction, thus causing accumulation of the toxic metabolic precursor of ethanol, acetaldehyde, and its derivative, acetoin (Ishikawa *et al.*, 1990). Both these compounds inhibit growth of *Z. mobilis* (Viikari, 1988). The production of acetaldehyde probably explains the remarkable finding (U. Kalnenieks *et al.*, in preparation) that cyanide markedly stimulates aerobic growth of *Z. mobilis*, while inhibiting respiration. This is attributed to increased availability of NADH (through not being oxidized by respiration) and enhanced reduction of acetaldehyde to ethanol.

One possibility that has been considered for the role of the respiratory chain (Pankova *et al.*, 1988) is respiratory protection (see also section 4.1). Another is that the production of inhibitory metabolites, like acetaldehyde, is a competitive growth strategy of aerated *Z. mobilis*. Thus, teleologically considered (U. Kalnenieks *et al.*, in preparation), *Z. mobilis* might prefer production of

substances inhibitory for other bacteria at the expense of rapid growth of its own biomass. Indeed, it is well established that *Z. mobilis* is inhibitory for other bacteria in interspecies conjugation. For anaerobic cultures, the competitive growth strategy of *Z. mobilis* might be based in part on the reported very high specific rates of ethanol production together with an ethanol tolerance exceeding that of many other microorganisms (Viikari, 1988). A similar strategy for aerobic growth would then imply high, 'excessive' respiration rates, and would lead to the observed low growth yields and self-inhibition.

3.10. Extremophiles

Extremophiles are a group of microorganisms that have attracted considerable attention as the gene products of these organisms are of commercial value in biotechnology. Extremophiles thrive under conditions of extreme pH, temperature, salt and hydrostatic pressure. Whilst the majority of extremophiles belong to the domain Archaea, Bacteria (eubacteria) are also represented in this group. Several genera of bacteria and Archaea have an aerobic lifestyle and respire using multiple terminal respiratory oxidases.

3.10.1. Bacteria

The thermophiles *Bacillus stearothermophilus* and *Thermus thermophilus* have relatively well-characterized respiratory systems. *B. stearothermophilus* respires using two terminal respiratory oxidases (Kusano *et al.*, 1996; Sakamoto *et al.*, 1997, 1999a,b; Nikaido *et al.*, 1998). A cytochrome caa_3-type cytochrome *c* oxidase in the haem–Cu superfamily is the main terminal oxidase. This is a SoxM-type oxidase that contains a cytochrome *c* moiety fused to subunit II (Kusano *et al.*, 1996). A cytochrome *cao'*-type oxidase is also present that is inhibited by potassium cyanide and is synthesized under dioxygen-limited conditions (Sakamoto *et al.*, 1997). Cytochrome c_{551} appears to be the natural substrate of this enzyme (Sakamoto *et al.*, 1997). This SoxB-type oxidase consists of two subunits encoded by the *cbaAB* operon and appears to constitute a unique subgroup of the haem–Cu family (Nikaido *et al.*, 1998). The enzyme has a high-spin haem O instead of haem A at the O_2-reducing binuclear centre (Sakamoto *et al.*, 1997, 1999a). Because SoxB-type oxidases have not been described in mesophilic bacteria, yet have been found in thermophilic microorganisms and Archaea, it has been proposed that SoxB-type oxidases may somehow be more suitable for bacteria growing under these extreme environmental conditions. For example, no SoxB-type oxidases have been found in the *B. subtilis* genome sequence which has 2 sets of SoxM genes encoding a caa_3-type cytochrome *c* oxidase and an aa_3-type quinol

oxidase (Sakamoto *et al.*, 1997, 1999a). Either cytochrome aa_3 or *cao'* can support aerobic growth of *B. stearothermophilus*, and mutants defective in the caa_3-type oxidase exhibit levels of NADH-dependent respiration that are close to those of wild-type cells. Information on whether these two oxidases are preferentially expressed depending on the dioxygen concentration is currently lacking.

In addition to the caa_3- and *cao'*-type cytochrome *c* oxidases, there is evidence in *B. stearothermophilus* for a third oxidase of the cytochrome *bd*-type (Sakamoto *et al.*, 1996, 1999b). This oxidase is not detected in the wild-type strain or in other thermophilic bacilli grown under high aeration or dioxygen-limited conditions. It is possible that these mutants can grow because repression of the *bd*-type oxidase has been relieved. Compared with other cytochrome *bd*-type oxidases (e.g. *E. coli*), the cytochrome *bd* from *B. stearothermophilus* has lower molecular masses for the two subunits and appears to be evolutionarily older.

The aerobic Gram-negative thermophilic bacterium *T. thermophilus* respires using two terminal oxidases, cytochromes caa_3 and ba_3 (Fee *et al.*, 1980, 1986, 1993; Hon-nami and Oshima, 1980; Keightley *et al.*, 1995). Cytochrome caa_3 is a two-subunit cytochrome *c* oxidase in the haem–Cu oxidase family (Fee *et al.*, 1986; Mather *et al.*, 1993). Cytochrome ba_3 is also a cytochrome *c* oxidase in this family (Zimmerman *et al.*, 1988) but has low (< 20%) similarity with most cytochrome *c*- and quinol-oxidizing terminal oxidases (Keightley *et al.*, 1995). It belongs to the SoxB cluster along with other phylogenetically distant oxidases already partially characterized, such as the *cao'*-type cytochrome *c* oxidase of *B. stearothermophilus* (Sakamoto *et al.*, 1997; Nikaido *et al.*, 1998), SoxABCD of *Sulfolobus acidocaldarius* (Anemuller and Schafer, 1990; Lubben *et al.*, 1992, 1994a,b) and cytochrome aa_3 of *Acidianus ambivalens* (Purschke *et al.*, 1997). Cytochrome ba_3 is found predominantly under reduced dioxygen tensions (Keightley *et al.*, 1995).

The crystal structures of the cytochrome aa_3-type cytochrome *c* oxidase of *Paracoccus denitrificans* (Iwata *et al.*, 1995) and bovine heart (Tsukihara *et al.*, 1996) have revealed two putative proton pathways referred to as the D- and K-pathways. The quinol and cytochrome c oxidases from *T. thermophilus* and a number of thermophilic Archaea such as *S. acidocaldarius* (Gleissner *et al.*, 1994, 1997) appear to lack conserved amino acid residues of both D and K pathways, and therefore it has been unclear whether or not they function as proton pumps. Kannt *et al.* (1998) performed proton-pumping measurements in *T. thermophilus* and showed that the cytochrome ba_3-type cytochrome *c* oxidase turnover is indeed coupled to the generation of an electrocurrent and proton pumping across the cell membrane. Because *T. thermophilus* lacks the D-pathway that has been shown to be indispensable for proton pumping in other haem–Cu-containing terminal oxidases, there must be mechanistic and/or structural variations to allow the *T. thermophilus* enzyme to pump protons.

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3.10.2. Archaea

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The energetics of alkaliphiles poses fascinating questions, particularly regarding their ability to generate a protonmotive force at very low external H^+ concentrations. Although the respiratory chains of such bacteria have been extensively studied, there is currently no evidence for special complexity or elaborate terminal branching of the respiratory chain in a typical alkaliphilic *Bacillus* (reviewed in Krulwich *et al.*, 1998). Nevertheless, in *Bacillus firmus* OF4, mutational loss of the high pH-inducible cytochrome caa_3 leads to a non-alkaliphilic phenotype. A second oxidase, cytochrome *bd*, is elevated in such *cta* mutants but does not support growth of the mutant on non-fermentable carbon sources even at near-neutral pH values (Krulwich *et al.*, 1998).

3.10.2. Archaea

In recent years considerable information has accumulated on archaeal respiratory proteins and oxidases (Castresana *et al.*, 1995; Lubben, 1995; Schafer *et al.*, 1996a,b). Whilst in most aerobic Archaea, *a*- and *b*-type cytochromes are found (Lubben, 1995), *c*-type cytochromes have been reported only for the halobacteria (Denda *et al.*, 1991, 1995). Terminal respiratory oxidase have been described in *Halobacterium salinarum* (Sreeramula *et al.*, 1998), *Natronobacterium pharaonis* (Scharf *et al.*, 1997), *Sulfolobus solfataricus* (Wakagi *et al.*, 1989), *Sulfolobus acidocaldarius* (Anemuller and Schafer, 1990; Lubben *et al.*, 1994a,b), and *Acidianus ambivalens* (Purschke *et al.*, 1997).

Two terminal respiratory oxidases in the thermophilic acidophile *S. acidocaldarius* have been described with novel structural features (Anemuller and Schafer, 1990; Anemuller *et al.*, 1992, 1993). The first, cytochrome aa_3, was shown to be part of a complex identified as the product of the *soxABCD* operon (Anemuller and Schafer, 1990; Lubben *et al.*, 1992, 1994a,b). The oxidase has four haem As and five different polypeptides and functions as a quinol oxidase. The SoxAB proteins (subunits I and II) are structurally related to the cytochrome *c* subunits I and II, and the SoxABCD complex is reminiscent of a functional fusion of an aa_3-type terminal oxidase with a *b*-type cytochrome like that operating in ubiquinone:cytochrome *c* reductase. The SoxABCD complex exhibits distinct differences from other known cytochrome aa_3-type oxidases (Anemuller and Schafer, 1990). Firstly, the oxidized form has absorption maxima at 421 and 597 nm and the reduced form at 439 and 601 nm. The oxidase consists of a single polypeptide with two haems A and two Cu ions and does not oxidize cytochrome *c* (Lubben *et al.*, 1992). The purified enzyme contains novel haem As. These have similar spectroscopic features to haem A, but have a hydroxyethylgeranylgeranyl side-chain instead of a hydroxyfarnesyl group. Possibly, these novel haems are cofactors binding to the binuclear reaction centres of Archael cytochrome oxidases (Lubben and Morand, 1994).

Subunit I encoded by *soxB* has been shown to catalyse the effective generation of a protonmotive force when reconstituted into lipid vesicles (Gleissner *et al.*, 1994, 1997) but this may simply be achieved by charge separation. As mentioned above, this oxidase lacks essential amino acids that form one of the two channels for proton translocation to the binuclear haem-a_3/Cu_B redox centre in H^+-pumping cytochrome *c* oxidases (Anemuller and Schafer, 1990; Lubben *et al.*, 1994b). The other proton-translocating complexes, NADH:coenzyme Q reductase (complex I) and the coenzyme Q:cytochrome *c* reductase (bc_1 complex), have not been found in aerobic Archaea.

An alternative second oxidase in *S. acidocaldarius*, a SoxM-type, has been described with high homology to haem–Cu oxidases. The SoxM oxidase is encoded by the gene cluster *soxEFGHIM* (Castresana *et al.*, 1995) and is a fusion between two central components of cytochrome oxidases, subunit I and subunit III. Supercomplex SoxM combines all features of a classical cytochrome bc_1 complex merged with a H^+ pumping terminal oxidase (Lubben *et al.*, 1994a,b; Castresana *et al.*, 1995; Lubben, 1995). The oxidase has two functional parts which, based on structural and redox potential analyses, are likely to pump two and four H^+, respectively, per oxidized quinol. Both contribute to the generation of Δp, but by different mechanisms and therefore there appears to be redundancy within this oxidase for the generation of Δp.

In *S. acidocaldarius*, both the SoxABCD and SoxM oxidases are expressed constitutively, do not respond to dioxygen tension, and are unaffected by the growth conditions used to study expression (Schafer, 1999). It is possible that these oxidases have different dioxygen affinities, but this remains to be proven experimentally. There is some evidence that a third oxidase exists in *Sulfolobus* which may involve a Fe–S cluster (Schafer, 1999). At present, the coexistence of parallel systems and the lack of specific inhibitors hamper the elucidation of their distinct physiological importance and properties (Schafer, 1999).

Other Archaea have been studied with respect to their terminal respiratory proteins. For example, *A. ambivalens*, an obligate chemolithotroph that grows at 80°C and pH 2.5, has a simple electron transport chain and seems to lack membrane-bound *b*-type cytochromes (Purschke *et al.*, 1997). *A. ambivalens* has a terminal quinol oxidase of the cytochrome aa_3-type that is inhibited by cyanide and quinolone analogues (Purschke *et al.*, 1997). This oxidase comprises five subunits encoded by genes in two operons (Purschke *et al.*, 1997). Both operons exist in duplicate on the genome. Only the haem-bearing subunit I (the *doxB* product) exhibits clear homology to other members of the haem–Cu superfamily of oxidases. Based on alignments and phylogenetic analysis of subunit I, this oxidase is considered to be located at the bottom of the phylogenetic tree, in the branch of the haem–Cu oxidases recently suggested to be incapable of proton pumping. This is also supported by the lack of essential amino acid residues delineating the putative H^+ pumping channel. *A. ambivalens* therefore appears to cope with its strongly acidic environment, the

consequent large proton gradient across the cytoplasmic membrane, and the need for proton extrusion, simply by an extremely high turnover of its terminal oxidase and chemical charge separation (Purschke *et al.*, 1997).

Halobacterium salinarum is a halophilic archaeon that has a novel cytochrome aa_3-type oxidase belonging to the haem–Cu oxidase superfamily (Sreeramula *et al.*, 1998), but it is more similar to eukaryotic cytochromes (Denda *et al.*, 1991). It is unknown whether this is a cytochrome *c* oxidase or a quinol oxidase. Based on sequence similarities this oxidase has been proposed to be H^+-pumping.

Natronobacterium pharaonis is a haloalkaliphilic aerobic Archaeon that respires via a cytochrome *c* oxidase with a cytochrome ba_3–Cu composition (Scharf *et al.*, 1997). This four-subunit cytochrome *c* oxidase is encoded by the *cbaDBAC* operon (Mattar and Engelhard, 1997). There is no spectroscopic evidence for a second oxidase, so the respiratory pathway appears to be unbranched. Based on sequence comparisons, the cytochrome ba_3-type haem oxidase is most closely related to the archaeal quinol oxidase SoxABCD and cytochrome ba_3 from *T. thermophilus*. This organism is the only Archeon where the presence of a membrane-bound cytochrome *c* has been found, thus supporting the role of this oxidase as a cytochrome *c* oxidase (Scharf *et al.*, 1997).

4. REASONS: OTHER ROLES FOR RESPIRATORY METABOLISM AND THE SPECIAL CASE OF CYTOCHROME *bd*

In this section, we examine some of the many aspects of bacterial physiology that depend, in many cases surprisingly, on respiration and particular oxidases. Attention is focused on cytochrome *bd*, a terminal oxidase widely distributed in Gram-positive and -negative bacteria. There are several notable features of its structure, assembly and function.

- At the protein level, it is completely unrelated to the haem–Cu ('mitochondrial') super-family of terminal oxidases, yet the sequences of the two polypeptides (CydA, CydB) of *bd*-type oxidases in several bacteria reveal that its structure is highly conserved (Osborne and Gennis, 1999).
- Assembly of cytochrome *bd*, at least in *E. coli* and *B. subtilis*, requires function of an ABC transporter, encoded by the *cydDC* operon. The transported substrate is unknown. A striking feature of *E. coli cydDC* mutants is that, not only cytochrome *bd*, but also periplasmic cytochromes *c* and b_{562}, also fail to assemble. This led us to propose (Poole *et al.*, 1993, 1994) that CydDC might export haem to the periplasm for the assembly of all these cytochromes, but some contradictory evidence has subsequently been presented (Goldman *et al.*, 1996a).

- Some oxidases of the *bd*-type have the highest affinities for dioxygen ever measured; in *E. coli* the K_m is around 5 nM, although in *Azotobacter vinelandii* the K_m is 1000-fold higher (see Table 1).
- Haem *d* forms a remarkably stable adduct with O_2, the so-called 650 nm form, which can be observed in growing cultures and intact cells (Poole, 1988; Junemann, 1997; Kavanagh *et al.*, 1998); its physiological significance is unknown (but see section 5).

Since *cydDC* mutants (defective in the ABC transporter) fail to synthesize cytochrome *bd*, and both *cydAB* and *cydDC* mutants exhibit stationary-phase loss of viability (Siegele *et al.*, 1996), and sensitivity to low iron (Cook *et al.*, 1998), and other stresses (see below), it is important to establish which of these defects are due to oxidase deficiency *per se* and which are due to loss of the transporter. *E. coli* K-12 *cydDC* mutants have a few (unexplained) deficiencies that are not shared by *cydAB* mutants. However, since *cydDC* mutants fail to assemble cytochrome *bd*, they also share all the phenotypes of *cydAB* mutants (Fig. 5).

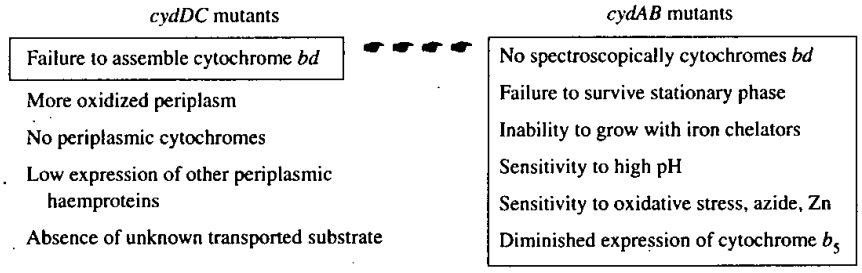

Figure 5 Relationships between the phenotypes of *cydDC* and *cydAB* mutants of *E. coli*. For references see the text, Goldman *et al.* (1996a,b), Siegele *et al.* (1996), and references therein.

4.1. Respiratory Protection

The concept of respiratory protection of dioxygen-labile functions, specifically nitrogenase activity in diazotrophic bacteria, was suggested by Dalton and Postgate 30 years ago (for a review, see Poole and Hill, 1997). Essentially, high, 'uncoupled' rates of respiratory dioxygen consumption are viewed as maintaining intracellular levels of dioxygen below a level that would be toxic. Given the notorious dioxygen sensitivity of nitrogenase, the ability of *A. vinelandii* to fix nitrogen while growing under conditions of air saturation, and

the assumed facile permeation of dioxygen through cell membranes, this is a remarkable mechanism. Although other means of protecting nitrogenase from dioxygen are known (references in Poole and Hill, 1997), *A. vinelandii* mutants lacking only cytochrome *bd* fail to grow diazotrophically unless the dioxygen concentration is reduced (Kelly *et al.*, 1990), demonstrating the importance of respiration mediated by that branch of the respiratory chain. Mutants defective in an alternative oxidase in the haem–Cu superfamily are unaffected. In *A. vinelandii*, cytochrome *bd* has only a moderate affinity for O_2, so the capacity to support very rapid respiration is thought to be critical. Operation of cytochrome *bd* in respiratory protection is envisaged (Bertsova *et al.*, 1998) as being coupled with use of a capsaicin-resistant NADH:UQ oxidoreductase that is not coupled to energy conservation. Bertsova *et al.* (1997) have also shown that cytochrome *bd* is capable of contributing to generation of the protonmotive force, but that the H^+/O ratio is only 1, as in the case of *E. coli* and *B. subtilis*. This, and the absence of a 'Q-cycle' in the cytochrome *bd*-terminated pathway, results in a five-fold lower ATP generation per dioxygen consumed than for the 'cytochrome *o*'-mediated pathway. This highly active, partially coupled pathway seems well suited to the respiratory protection function.

Some doubts have been expressed about the ability of respiratory protection to allow aerobic nitrogen fixation. For example, Linkerhägner and Oelze (1995) measured ATP levels in *A. vinelandii* cultures exposed to long-term increases in dioxygen concentration and showed that, unlike the isogenic wild-type strain, a Cyd^- mutant was unable to recover from this stress, as judged by the failure of cellular ATP levels to recover to pre-oxygenation levels. The mutant also failed to increase respiration rates to meet the increase in dioxygen concentration. As Linkerhägner and Oelze (1995) point out, it appears paradoxical that the 'uncoupled' respiratory branch terminating in cytochrome *bd* should be required for maintaining energy status. Early schemes for 'sites' of oxidative phosphorylation in *A. vinelandii* cited by Linkerhägner and Oelze (1995) require major re-evaluation, but it seems clear that proton translocation upstream of the terminal cytochrome *bd* branch may make major contributions to Δp and ATP generation. Linkerhägner and Oelze conclude that 'regeneration of ATP rather than consumption of oxygen' is important in protecting nitrogenase activity. The measurements of ATP levels presented by these authors may, however, be a misleading measure of energy status, particularly in the absence of assays of ADP, AMP and the 'energy charge'. As in other areas of understanding the influence of dioxygen on bacterial physiology, sensitive means of assaying dioxygen *in vivo* and in real time are urgently needed to evaluate the respiratory protection hypothesis.

It was suggested by Jones two decades ago (see Poole and Hill, 1997) that cytochrome *bd* may be organized in the *A. vinelandii* membrane such that its active site faces the periplasm. Such an orientation might be expected to

maximize the effectiveness of dioxygen scavenging and may also explain the different dioxygen affinities measured in intact cells and everted vesicles (Poole and Hill, 1997). Recently, Osborne and Gennis (1999) have re-analysed numerous cytochrome *bd* sequences and proposed a revised topology for subunit I (by adding two transmembrane helices) that repositions H19, the putative ligand for cytochrome b_{595}, close to the periplasmic edge of the membrane. This suggests that the O_2-reactive site may indeed be outward-facing.

The respiratory protective role of cytochrome *bd* in *Klebsiella pneumoniae* has been covered in section 3.3. In *R. meliloti* and *Bradyrhizobium japonicum*, cytochrome *cbb'* appears critical in this role (section 3.4). In *Azorhizobium caulinodans*, both cytochromes *bd* and *cbb'* act as oxidases at low dioxygen tensions, and either seems to offer respiratory protection to allow nitrogen fixation at 50% of wild-type levels (see section 3.4 and Poole and Hill, 1997).

Very recently, Flores-Encarnación *et al.* (1999) have described the respiratory chains of *Acetobacter diazotrophicus*, an obligately aerobic diazotroph and plant endophyte. Cytochrome '*ba*' was identified as a putative oxidase in diazotrophic cultures; this haemprotein had spectral similarities to cytochrome aa_3, but with slightly blue-shifted maxima and with considerable enhancement of a 589 nm signal in the presence of cyanide. It is perhaps equivalent to 'cytochrome a_1'; the presence of haem A in membranes was confirmed. Repression of nitrogen fixation in well-aerated cultures by NH_4^+ also depressed respiration, including a 10-fold decrease in cytochrome *ba* levels. In such NH_4^+-supplemented cells, cytochrome *bd* appears to be a major oxidase, at least based on spectroscopic quantitation. Further work is required to confirm the oxidase roles of the haemproteins identified and to demonstrate the putative respiratory protection function of cytochrome *ba*.

4.2. Requirements for Cytochromes *bd* and *bo'* at low Δp

During aerobic respiration, membrane-associated protein complexes catalyse redox reactions which promote the transfer of electrons to dioxygen and cause the efflux of protons (H^+) across the cell membrane. Some bacteria, however, use Na^+ ions in addition to, or instead of protons, to couple exergonic reactions with endergonic reactions in the membrane (Skulachev, 1989). A ΔpNa^+ may be generated in a number of ways (Dimroth, 1994) including using the Δp via a Na^+/H^+ antiport, by a decarboxylase, or an NADH oxidase Na^+ pump (Dimroth, 1994). The Na^+-ion gradients may be used to drive solute transport, flagellar motion or ATP synthesis.

Skulachev (1985, 1989) postulated that adaptation of bacteria to growth at high [Na^+] involves substitution of Na^+ for H^+ as a coupling ion. Furthermore, it was found that adaptation of *E. coli* to alkaline pH growth conditions is accompanied by induction of a primary Na^+ motive NADH-quinone reductase

and terminal oxidase activities that generate ΔpNa^+ by Na^+ extrusion from the cell (Avetisyan *et al.*, 1989, 1991, 1992). Skulachev and co-workers have studied the roles of the two terminal oxidases, cytochrome *bd* and *bo'*, in this process. A Cyd^- mutant grew slowly or not at all under conditions of low Δp; e.g. at alkaline pH or in the presence of the protonophore pentachlorophenol (PCP). Using inside-out subcellular vesicles from Cyd^- mutants, Avetisyan *et al.* (1992) demonstrated that these mutants could not transport Na^+ when succinate was oxidized in the presence of a protonophore. The same vesicles were found to transport Na^+ when NADH is oxidized as if a Na^+-motive NADH-quinone oxidase were operative. In contrast, a mutant lacking the cytochrome *bo'* oxidase grew at low Δp conditions as fast as an isogenic wild-type strain containing both cytochromes *bd* and *bo'*. Growth in the presence of a protonophore induced a strong increase in the level of cytochrome *bd* in a wild-type strain. It was concluded that cytochrome *bd* operates as a Na^+ pump in *E. coli* grown under conditions unfavourable for the H^+ cycle.

More recent work by this group (Bogachev *et al.*, 1995) has shown that not only PCP, but also several other protonophores (e.g. carbonyl cyanide *m*-chlorophenylhydrazone, CCCP; 2,4-dinitrophenol, DNP; 4,5,6,7-tetra-chloro-2-trifluoromethylbenzimidazole, TTFB), elevated cytochrome *bd* expression. Sodium ions added to the growth medium also caused an increase in the cytochrome *bd* level, and the inducing effects of uncouplers were much more pronounced in the presence of high concentrations of sodium. Mutations in *arcAB* – a known regulator of cytochrome *bd* expression – prevented the induction of cytochrome *bd* by uncouplers, but had no effect on induction by Na^+ (Bogachev *et al.*, 1995).

The hypothesis that *E. coli* is able to use Na^+ as a coupling ion at alkaline pH predicts that growth at high pH should be relatively insensitive to a protonophore. Indeed, Avetisyan *et al.* (1993) have reported that *E. coli* can grow on succinate by oxidative phosphorylation at pH 8.6 in the presence of an uncoupler. However, Fillingame and Divall (1999) have questioned this hypothesis by showing that growth of *E. coli* in buffered medium at pH 8.6–8.8 was completely inhibited by 20 μM CCCP, suggesting that growth was still dependent on Δp.

The idea that the respiratory chain may function as a sodium pump under certain growth conditions (e.g. alkaline pH) has been reported also in other bacterial genera. For example, Na^+-pumping respiratory chains have been identified in marine species of *Vibrio* (Tokuda and Unemoto, 1982) and a halophilic bacterium (Ken-Dror *et al.*, 1986). In these bacteria, the NADH:quinone oxidoreductase has been identified as a respiratory-driven Na^+ pump, but the terminal oxidase of these bacteria remains a H^+ pump. A Na^+-motive terminal oxidase has been reported in the marine akalotolerant *Bacillus* BTU (Semeykina *et al.*, 1989; Semeykina and Skulachev, 1992), and *Vitreoscilla* generates a respiratory-driven $\Delta\mu_{Na+}$ (i.e. a transmembrane

difference in electrochemical Na^+ potential) (Efiok and Webster, 1990a,b; Park *et al.*, 1996). Two major respiratory electron transport proteins, NADH dehydrogenase (NADH:quinone oxidoreductase), and cytochrome *bo′*, are candidates in this bacterium for the electrogenic Na^+ pumping that mediates $\Delta\mu_{Na+}$ formation. Efiok and Webster (1990a) demonstrated that the rate of menadiol oxidation by cytochrome *bo′* was enhanced specifically by Na^+ but not Li^+. Furthermore, purified cytochrome *bo′*, reconstituted into Na^+-loaded liposomes in the right-side-out orientation, catalysed a net Na^+ extrusion that was inhibited by KCN. These results are consistent with the *Vitreoscilla* cytochrome *bo′* being a redox-driven Na^+ pump. This sodium gradient has also been shown to be coupled to ATP synthesis (Efiok and Webster, 1992).

4.3. Stationary Phase Survival

Bacterial cells respond to starvation by turning on a reversible programme of gene expression enabling them to survive prolonged periods of nutrient deprivation. Even in bacteria like *E. coli* that do not produce spores or other highly differentiated structures, these developmental processes allow starved cells to resist many different environmental stresses. Similarly in the stationary phase, which cannot be simply equated with starvation, bacteria undergo developmental changes that enhance survival. Amongst these are increased resistance to oxidative damage and storage of high-energy compounds such as glycogen and polyphosphate to provide the dormant cells with energy reserves. Many of the changes that accompany the onset of the stationary phase are directed by σ^s (the 's' indicating stationary phase), encoded by *rpoS* (Goodrich-Blair *et al.*, 1996).

Amongst the 20 or so genes whose expression is σ^s-dependent (a list of σ^s-dependent genes is given by Goodrich-Blair *et al.*, 1996) are some involved in respiration and energy metabolism. The *app*Y regulatory gene that determines expression of the third oxidase, cytochrome *bd*-II, was covered in section 3.1.2. It is not clear if this oxidase is especially important in the stationary phase; experiments with mutants lacking both cytochromes *bo′* and cytochrome *bd*-I are needed. However, both *cydAB* and *cydDC* mutants lose viability, defined as culturability on solid media at 37°C, when subjected to prolonged incubation on reaching the stationary phase. Siegele *et al.* (1996) have reported that *cydC* mutants (described in their paper as *surB*) exhibit a more pronounced phenotype. Increased expression of the alternative oxidase, cytochrome *bo′*, from a multicopy plasmid suppressed these growth defects, presumably reflecting a requirement for a certain level of dioxygen consumption, irrespective of the nature of the oxidase. Siegele *et al.* (1996) tested the idea that the lack of an oxidase might raise superoxide levels, but overexpression of SOD did not suppress the stationary phase exit defect. What is puzzling

is that the aerobic growth defects of a *cydC* mutant are more severe than those of a *cydC* Δ*cydAB* mutant, suggesting that the defective or incomplete oxidase polypeptides produced in the *cydC* mutant are detrimental. More work is needed to explain these complex phenotypes.

During prolonged starvation and stasis, *arcA* mutants fail to decrease the synthesis of Krebs-cycle enzymes and show elevated rates of respiration and metabolic activity. Because this phenotype can be rescued by overexpression of superoxide dismutase (Nystrom *et al.*, 1996), it is suggested that decreased respiratory activity in stasis is important in avoiding the damaging effects of dioxygen radicals.

4.4. CydDC- and CydAB-Dependent Redox Biochemistry in the Periplasm

Goldman *et al.* (1996a) made the important finding that the periplasmic reducing environments of *cydDC* and *cydAB* mutants are different: using the periplasmically located Ccl2 protein of *Rhodobacter capsulatus* as redox sensor, *cydDC* mutants were shown to be defective in the reduction environment of the periplasm; i.e. to have a periplasm even more oxidized than in wild-type cells. Being separated from the external environment only by a somewhat permeable outer membrane, the periplasm is vulnerable to changes in pH, osmolarity and the presence of small molecules and ions. Nevertheless, proteins that function in, or are in transit through, the periplasm must be correctly folded and stabilized. A number of periplasmic proteins have been identified which act as folding catalysts. These include the protein disulfide isomerases that carry out thiol–disulfide exchanges, and peptidyl prolyl isomerases (PPI). Several Dsb (disulfide bond) enzymes have now been identified: DsbA and DsbB act as strong thiol:disulfide oxidants and DsbD and DsbE act as thiol:disulfide reductants along with three PPIs. Failure to control the periplasmic reducing environment could explain several aspects of the Cyd^- phenotype. For example, cytochrome *c* biosynthesis in the periplasm depends upon a mechanism for maintaining in the reduced state the correct pairs of cysteine residues to which the haem is to be attached covalently (Kranz *et al.*, 1998). Furthermore, such an imbalance might affect iron transport through the periplasm by affording an environment in which Fe(III) bound to incoming siderophores and chelators cannot be reduced (see section 4.5). The consequences of inappropriate Dsb chemistry may be profound and unexpected, as illustrated by the recent report that *dsbA* mutants are defective in LPS structure, fimbriation and biofilm formation (Genevaux *et al.*, 1999).

At least two scenarios can be envisaged by which mutations in *cyd* genes affect the periplasmic reducing environment (Fig. 6). First, it is tempting to speculate that CydDC exports to the periplasm some component of the redox

Figure 6 Highly simplified view of the relationships between periplasmic redox reactions, cytochrome assembly, and respiration in *E. coli*. The ABC-type transporter is the CydD/CydC heterodimer, which probably exports to the periplasm an unidentified substrate required for the assembly of the oxidase cytochrome *bd* and the soluble periplasmic cytochromes b_{562} and *c*. CydAB is a quinol oxidase; other components of the respiratory chain (boxed), such as the other oxidase cytochrome *bo'*, are not shown. The DsbB/DsbA couple act in concert to oxidize proteins outwardly transported by the Sec pathway or ABC transporters (perhaps including CydDC). The reported requirement of the respiratory chain for oxidation of DsbB is indicated. In *cydDC*, but not *cydAB*, mutants, the periplasm is more oxidized. OM = outer membrane; P = periplasm; CM = cytoplasmic membrane; c = cytoplasm. 'Diamonds' in CydAB represent the three haems: cytochrome b_{558} in CydA and cytochromes b_{595} and *d* shared between the two subunits.

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4.5. Iron metabolism

E. coli Cyd$^-$ mutants (*cydAB* or *cydDC*) are inhibited by the presence of Fe(III) chelators, including enterochelin secreted by neighbouring cells (Cook *et al.*, 1998). The preferential inhibition of Cyd$^-$ mutants by these chelators is not due to a decrease in expression, activity or assembly of cytochrome *bo'*, the major alternative oxidase. The complexity of iron transport systems poses challenges to unravelling this phenotype, but it is possible that the major changes in periplasm biochemistry evident in Cyd$^-$ mutants (section 4.4) in some way affect Fe(III)-siderophore recognition or transfer across the periplasm. Cyd$^-$ mutants of *A. vinelandii* also display a very complex phenotype that includes sensitivity to iron deprivation, metal toxicity, stationary phase and oxidative stress (S. Edwards, S. Hill, B.W. Bainbridge and R.K. Poole, unpublished).

Another curious link between respiratory metabolism and iron metabolism is that revealed by studies of mutants in the *cycHJKL* operon of *Rhizobium leguminosarum*. These genes are involved in the biogenesis of cytochrome *c*, but *cyc* mutants are pleiotropically defective. In particular, Cyc$^-$ strains lose the high-affinity iron acquisition system owing to the inability to export siderophores (Yeoman *et al.*, 1997). One possibility is that the biosynthesis of siderophores may require an electron transfer step involving a *c*-type cytochrome. Alternatively, the lack of cytochrome *c* apoprotein might raise the level of haem in the periplasm, which is 'sensed' as an indication of iron sufficiency, leading in turn to down-regulation of siderophore production. It is probable that the explanation, when found, will shed light on the links between iron uptake and aerobic respiration now identified in several bacterial genera.

Figure 6 Highly simplified view of the relationships between periplasmic redox reactions, cytochrome assembly, and respiration in *E. coli*. The ABC-type transporter is the CydD/CydC heterodimer, which probably exports to the periplasm an unidentified substrate required for the assembly of the oxidase cytochrome *bd* and the soluble periplasmic cytochromes b_{562} and *c*. CydAB is a quinol oxidase; other components of the respiratory chain (boxed), such as the other oxidase cytochrome *bo'*, are not shown. The DsbB/DsbA couple act in concert to oxidize proteins outwardly transported by the Sec pathway or ABC transporters (perhaps including CydDC). The reported requirement of the respiratory chain for oxidation of DsbB is indicated. In *cydDC*, but not *cydAB*, mutants, the periplasm is more oxidized. OM = outer membrane; P = periplasm; CM = cytoplasmic membrane; c = cytoplasm. 'Diamonds' in CydAB represent the three haems: cytochrome b_{558} in CydA and cytochromes b_{595} and *d* shared between the two subunits.

4.6. Oxidative Stress

Aerobic respiration not only provides energy to the cell, but also produces reactive dioxygen species (ROS). ROS include both dioxygen radicals (e.g. the superoxide and hydroxyl radicals), and nonradical reactive compounds like H_2O_2 (Demple, 1991). Aerobic respiration in *E. coli* accounts for about 87% of the H_2O_2 produced *in vivo* (González-Flecha and Demple, 1995) and metabolic O_2^- production in growing *E. coli* cells is due primarily to the 'leakage' of electrons by autooxidation of components of the respiratory chain (Imlay and Fridovich, 1991). NADH dehydrogenase II and fumarate reductase have been particularly implicated (for a review, see Storz and Imlay, 1999).

To cope with such oxidative stress, *E. coli* has at least two inducible defence regulons (Farr and Kogoma, 1991): the *soxRS* regulon that responds to increased levels of O_2^- (Greenberg *et al.*, 1990; Nunoshiba *et al.*, 1992), and the *oxyR* regulon that responds to H_2O_2 stress (Storz *et al.*, 1990). Superoxide dismutases, which scavenge O_2^-, and catalases and peroxidases, which scavenge H_2O_2 are up-regulated to provide the necessary defence. However, a better strategy to cope with oxidative stress may be to keep the intracellular partial pressure of O_2 low, thereby preventing excessive one-electron reduction of dioxygen (Skulachev, 1994). To date, the role of terminal respiratory oxidases in this process, which are very effective at keeping the intracellular partial pressure of dioxygen low, have not been fully assessed.

Previous work by Wall *et al.* (1992) has shown that both cytochrome *bo′* and cytochrome *bd* mutants are equally sensitive to H_2O_2 stress, suggesting that they might play a role in protecting cells from oxidative stress generated during aerobic respiration. Goldman *et al.* (1996b) have reported that both the temperature-sensitive and stationary-phase defects of Cyd$^-$ mutants can be alleviated by the addition of exogenous catalase and reducing agents. These authors further proposed that cytochrome *bd* protects cells from oxidative damage by reducing the levels of ROS directly or indirectly.

The production of superoxide radicals and H_2O_2 by membrane vesicles has been observed *in vitro* when the aerobic respiratory chain is blocked by inhibitors (Imlay and Fridovich, 1991; González-Flecha and Demple, 1995). The primary effect of blocking the electron transport chain is an enhanced production of superoxide radicals that subsequently dismutate to H_2O_2. These observations suggest that in the absence of either cytochromes *bd* or *bo′*, cells may overproduce ROS. However, Siegele *et al.* (1996) have demonstrated that the total superoxide levels produced by Cyd$^-$ strains and wild-type strains are indistinguishable. Based on their work, it does not appear that Cyd$^-$ mutants suffer oxidative stress through an overproduction of superoxide. However, these results do not rule out the possibility that *cyd* or *cyo* mutants may be overproducing H_2O_2 and/or are unable to remove H_2O_2.

To study the effect of H_2O_2 in greater detail, we (A. Lindqvist, J. Membrillo-Hernández, R.K. Poole and G.M. Cook, in preparation) have conducted experiments to measure intracellular H_2O_2 in *cyo* and *cyd* mutants using a Φ(*katG–lacZ*) fusion that will be induced on exposure to low levels of H_2O_2. Cyd⁻ mutants experience greater intracellular H_2O_2 stress than do wild-type and *cyo* cells. Interestingly, no induction of Φ(*katG–lacZ*) by H_2O_2 was observed in a *cyo* mutant. These results may reflect the ability of cytochrome *d* to react with H_2O_2 and form a stable complex (Poole and Williams, 1988). An alternative hypothesis is that, in the absence of cytochrome *bd*, the level of cytochrome *bo′* is elevated to compensate and that intracellular levels of H_2O_2 rise as a result of operation of this oxidase. Hassan and Fridovich (1978) have reported that full expression of catalase (*katG*) and MnSOD appear to be dependent on a functional electron transport chain in *E. coli*.

4.7. Nitric Oxide and Nitrosative Stress

Nitric oxide (NO) is a potent inhibitor of many cell functions, including respiration catalysed by terminal cytochrome and quinol oxidases. Respiration in *E. coli* is sensitive to micromolar concentrations of NO; and the transient, almost complete, inhibition of respiration elicited by NO markedly increases as dissolved dioxygen tension in the medium decreases (Yu *et al.*, 1997). There is currently no evidence that either oxidase is preferentially useful in resisting NO, in contrast to the effects of cyanide, to which cytochrome *bd* is notably resistant (Table 1). Cytochrome *bd* reacts with nitrite and trioxodinitrate to form a spectrally distinctive nitrosyl complex (Hubbard *et al.*, 1985) but might be expected to be less sensitive to NO, since it lacks the NO-reactive Cu atom identified in studies of the mitochondrial and other haem–Cu oxidases (Torres *et al.*, 1998). At low dioxygen concentrations (< about 50 μM of O_2), mutation of either oxidase slightly increases the period of transient inhibition (T.M. Stevanin and R.K. Poole, in preparation), suggesting that it is total electron flux to dioxygen, rather than the activity of a specific oxidase, that determines NO sensitivity. However, respiration of an *hmp* mutant defective in synthesis of the flavohaemoglobin Hmp is extremely NO-sensitive, demonstrating the role of this NO-inducible and -detoxifying protein (Poole and Hughes, 2000) in protection of respiration *in vivo*.

4.8. Pathogenicity

In many pathogenic bacteria, the diversity of respiratory pathways and the regulation of these pathways in response to changes in the organism's environment have not been elucidated. Recent work by Way *et al.* (1999) has

demonstrated a role for a terminal respiratory oxidase in bacterial virulence. A positive correlation was demonstrated between cytochrome *bd* expression and *Shigella flexneri* virulence as represented by the size of bacterial plaques formed, intracellular survival and lethal doses in intranasally infected mice. These authors propose that, because the lipopolysaccharide (LPS) layer is unaltered in cytochrome *bd*-deficient mutants, elimination of cytochrome *bd* could represent a novel form of attenuation in the development of live vaccines for pathogens with protective immunity (Way *et al.*; 1999). Cytochrome *bd* may be particularly relevant to those bacteria that inhabit the gastrointestinal tract that becomes progressively limited in dioxygen distal to the stomach.

Inspection of the recently determined genome sequence of *Mycobacterium tuberculosis* reveals the existence of genes (*cydABCD*) that may encode cytochrome *bd* (Cole *et al.*, 1998). Growth of mycobacteria at low dioxygen tensions that may result in the induction of cytochrome *bd* has been shown to enhance the cellular invasion of *Mycobacterium avium* (Bermudez *et al.*, 1997). In terms of the characterization of respiratory oxidases in mycobacteria, only *Mycobacterium leprae* has been characterized in detail (Ishaque, 1983, 1984, 1990, 1992). *M. leprae* is a microaerophilic bacterium requiring 2.5% dioxygen and 10% CO_2 to grow. NADH oxidation by this strain is only partially inhibited by KCN (50%) and this cyanide-resistant respiration has been attributed to cytochrome *bd*. Cytochrome *bo'* has also been detected in this bacterium (Mori *et al.*, 1985).

Cytochrome *bd* has been shown to be important in antibiotic resistance. Macinga and Rather (1996) have shown that mutations in *aarD*, a *cydD* homologue required for a functional cytochrome *bd* oxidase in *Providencia stuartii*, caused a 32-fold increase in resistance to the antibiotic gentamicin. The loss of cytochrome *bd* in this strain would be predicted to affect electron transport and potentially lead to a reduction in Δp, thereby decreasing the uptake of gentamicin. Other studies have demonstrated that the uptake of aminoglycosides such as gentamicin is dependent on the presence of a functional electron transport chain (Taber *et al.*, 1987).

A *Staphylococcus aureus* mutant defective in long-term starvation was shown to have a transposon mutation in a gene homologous to *ctaA* of *Bacillus subtilis*, which encodes haem A synthase (Clements *et al.*, 1999). The mutant lacked spectroscopically detectable cytochrome aa_3, had increased resistance to aminoglycoside antibiotics, and reduced production of haemolysin and toxic shock syndrome toxin 1. The poor recovery from starvation could be partially rescued by exogenous catalase, indicating a role, possibly indirect, of the oxidase in oxidative stress responses. The mutant showed a dramatic increase over the wild type in a band in CO difference spectra at 630 nm; its identity is not known, but a similar feature has been seen in *H. pylori* (Marcelli *et al.*, 1996).

4.9. Barotolerant Growth

Deep sea barophilic and barotolerant bacteria are adapted to life under conditions of extremely high pressure. In one barophilic organism, a pressure-regulated operon was found, downstream of which were found open reading frames (ORF3, ORF4) homologous to the *cydDC* operon of *E. coli*. This led to the finding that an *E. coli cydD* mutant is both temperature-sensitive (as previously reported) and pressure-sensitive (Kato *et al.*, 1996). At 0.1 MPa and 37°C, the *cydD* mutant did not grow, but introduction of ORF3 and ORF4 on a plasmid restored growth fully. At 30 MPa and 30°C, the *cydD* mutant growth was markedly poorer than the wild-type strain, but again introduction of ORF3 and ORF4 restored growth fully. Spectroscopic analysis of membranes confirmed restoration of cytochrome *bd* in the *cydD* mutant by ORF3. This aspect of the pleiotropic Cyd^- phenotype deserves further attention.

5. REGULATION OF OXIDASE SYNTHESIS AND FUNCTION

Maps of electron transport pathways such as those in Figs 2–4 disguise the fact that multiple parallel pathways, such as those to various terminal oxidants, probably rarely coexist in the bacterial cell. Such pathways are better thought of as menus, selection from which is made by intricate control mechanisms, generally operating at the level of gene transcription. The adaptability of bacterial respiration to dioxygen at the 'aerobic–anaerobic interface' has been repeatedly emphasized in the literature and many reviews exist; particularly useful is that of Sawers (1999).

The best-studied transcriptional regulators are Fnr and ArcA in *E. coli*. Fnr appears to sense dioxygen directly through an iron–sulphur cluster in the protein. The other well-studied regulator is ArcA whose phosphorylation state and activity is controlled by the membrane-associated histidine kinase ArcB. Further discussion of these regulators and their homologues and relatives in other bacteria is beyond the scope of this review. What is clear is that the special roles played by certain respiratory chain components is reflected in their mode of regulation. A few examples will suffice.

Regulation of cytochrome *bd* expression in *E. coli* is achieved by the interacting effects of Fnr and ArcA/B. ArcA activates *cydAB* gene expression at low dioxygen tensions. As dioxygen tension falls further, Fnr is activated and represses *cydAB* expression (Tseng *et al.*, 1996). Recent work has identified two *cydAB* promoters, but the roles played by Fnr and ArcA have not been fully elucidated. Lynch and Lin (1996) found three sites for ArcA, one of which (III) was located downstream of the previously identified *cydAB*.

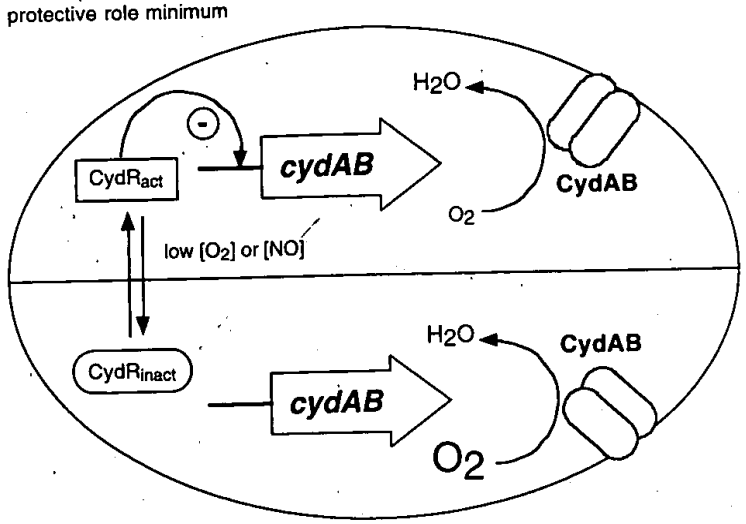

Figure 7 Model of regulation of *cydAB* expression by CydR and respiratory protection. CydR is an Fnr homologue and a highly sensitive monitor of cytoplasmic dioxygen, as anticipated for continued operation of nitrogenase under highly aerobic growth conditions. During growth under microaerobic conditions, intracellular dioxygen concentrations are sufficiently low to allow nitrogenase function and CydR will be active, repressing *cydAB* expression and synthesis of cytochrome *bd* (CydAB). Under conditions of stress imposed by high dioxygen, the repressed levels of cytochrome *bd* may not maintain the essentially anoxic state of the cytoplasm that is required for nitrogenase and CydR will be inactivated; this in turn derepresses cytochrome *bd* synthesis, which provides respiratory protection. The transitions between active and inactive CydR are brought about by NO as well as dioxygen.

promoter P_1 (P1). A second promoter was found downstream of this site, but could not be detected by analysis of RNA extracted from aerobically grown cells, suggesting that *cydAB* P1 is used preferentially under such conditions. It was suggested that ArcA-P (i.e. the active phosphorylated form) bound at site III activates *cydAB* anoxically when Fnr prevents transcription from P1 (Lynch and Lin, 1996). Subsequently, Cotter *et al.* (1997) demonstrated that a single site for ArcA-P upstream of promoter P1 was sufficient for activation of *cydAB* expression. Two sites for Fnr were found, one at the start of *cydAB* transcription at P1, and another centred 53.5 bp upstream of the +1 site of P1. Thus, collectively, ArcA and Fnr afford maximal *cydAB* expression in *E. coli*

growing in microaerobic environments, consistent with the finding that this quinol oxidase has a remarkably high affinity for dioxygen (D'mello *et al.*, 1996a). The match between functional capabilities of an individual component and regulation is, however, only approximate. For example, the extraordinarily high dioxygen affinity of cytochrome *bd* in *E. coli* (section 3.1.2; Table 1) would not be anticipated from studies that show that *cydAB* transcription is maximal at 7% air saturation (Tseng *et al.*, 1996); i.e. about 15 μM of O_2. We must assume that the complexities of function cannot be fully appreciated by such simplistic considerations.

The situation in *A. vinelandii* is strikingly different. Here, transcription of the *cydAB* operon, encoding the two subunits of cytochrome *bd*, is repressed by CydR (Wu *et al.*, 1997), an Fnr homologue. Mutation of CydR causes elevation of oxidase synthesis. Cytochrome *bd* levels as well as cytochrome *bd*-specific mRNA increase in a wild-type strain when dioxygen concentration increases under non-nitrogen-fixing conditions (D'mello *et al.*, 1996b; Wu *et al.*, 1997). The converse is true in *cydR* mutants; i.e. the cytochrome *bd* concentration increases sharply when dioxygen availability decreases. The aerobically purified CydR protein can be reconstituted into an active form, as can that of *E. coli* Fnr, and in this state binds to target sequences in the *cydAB* promoter now identified by footprinting studies and similarity with Fnr boxes (Wu *et al.*, 2000). Like Fnr, CydR is sensitive to dioxygen, but apparently much more so (Wu *et al.*, 2000). This observation is perhaps not surprising given the requirement in this organism that the cytoplasmic dioxygen tension should be maintained at very low levels. A model showing how CydR regulates *cydAB* expression is presented in Fig. 7.

Several lines of evidence indicate that cysteine-rich motifs of metal-binding proteins and redox-sensitive metal clusters of metalloproteins are natural biosensors not only of O_2 but also of NO. We have recently shown for the first time that CydR, a member of the Fnr family, is inactivated by NO as well as dioxygen (Wu *et al.*, 2000). The mechanism of this inactivation needs further study and the physiological function of the effects of NO on CydR, if any, are unclear. However, although *A. vinelandii* is not itself a denitrifying bacterium, it inhabits environments where other bacteria produce NO as an intermediate in this pathway. NO may derepress cytochrome *bd* so that nitrogenase is protected by respiration and able to exploit the end-product of denitrification, namely dinitrogen.

The low levels of cytochrome *bd* at low dioxygen tensions in wild-type cells are presumably due to repression by CydR, but levels of cytochrome *bd* under low aeration in *cydR* mutants are significantly higher than those in both wild-type and the *cydR* mutants under high aeration (D'mello *et al.*, 1996b). This suggests that there may be another regulator that represses the expression of cytochrome *bd* under high aeration. So far, only the *cydAB* operon has been unequivocally shown to be regulated by CydR. However, increased activity of

NADH:ubiquinone oxidoreductase that is insensitive to capsaicin (i.e. NDHII) is co-induced with cytochrome *bd* in a *cydR* mutant (Bertsova *et al.*, 1998), and the O_2-sensitive phenotype of a *nifU* mutant is corrected by the introduction of a *cydR* mutation (Hill *et al.*, 1999). An unexplained phenotype of *cydR* mutants is their inability to grow under conditions of low aeration (Kelly *et al.*, 1990; Wu *et al.*, 1997). A likely explanation is that one or more genes required for microaerobic growth are CydR-regulated.

The precise signal sensed by ArcB is unknown. It appears not to be dioxygen but is somehow responsive to redox and metabolic status of the cell, including the levels of NADH and lactate (references in Sawers, 1999). The Arc system may be more global than hitherto proposed, being involved in plasmid transfer and replication, and bacterial ageing. To what extent the respiratory apparatus that responds to Arc is involved in these disparate aspects of bacterial physiology remains to be learned.

An issue that has been little addressed is the question of how electrons are distributed between the available parallel pathways. When different electron acceptors are available, the controlled transport of one or more into the cell might be important, as has been suggested for nitrate in *P. denitrificans* (references in Ferguson, 1998). When only dioxygen is considered as oxidant, rate-limiting steps in the electron transport pathways might distribute electrons between different branches that terminate in different oxidases. For example, availability of quinone or cytochrome *c* might determine whether quinol oxidases or cytochrome *c* oxidases are prominent in dioxygen reduction. When two or three oxidases share a common electron donor, as the oxidases share ubiquinol in *E. coli*, control over electron flux is presumably determined by the amounts of each oxidase – determined largely by transcriptional regulation – and their affinities for ubiquinol and dioxygen. Yet another level of control is suggested by measurements of respiration catalysed by cytochrome *bd*: at dioxygen concentrations exceeding those giving V_{max}, respiration rate declines, suggestive of substrate inhibition (Poole, 1994; D'mello *et al.*, 1996a). Such an inactive complex might involve ligand binding at both haems in the binuclear centre, or perhaps increased occupancy of the 'oxygenated' cytochrome *d* (d_{650}), which appears to be inactive in oxidase turnover in both *E. coli* (Junemann, 1997) and *A. vinelandii* (Kavanagh *et al.*, 1998).

6. CONCLUDING REMARKS: TRUE REDUNDANCY OR IGNORANCE?

The primary functions of *multiple* terminal respiratory oxidases appear to be (a) termination of a respiratory chain with an oxidase that has appropriate attributes of dioxygen affinity, turnover rate, and energy conservation by

proton translocation, and (b) in a small number of diazotrophs, respiratory protection of a dioxygen-sensitive nitrogenase. Are there any situations where there appears to be no physiological reason for the presence of multiple oxidases?

One example of this appears to be provided by the symbiotic nitrogen-fixing bacterium *Azorhizobium caulinodans* (previously *Rhizobium* ORS571) which we will consider in more detail here as a possible example of extreme respiratory complexity but which also reveals potential pitfalls in this type of analysis. Kaminski *et al.* (1996) state that *A. caulinodans* 'uses at least five terminal oxidases, including cytochrome aa_3 (cytaa_3) cytcbb_3, and an alternative *a*-type cytochrome, which are specific for cytc as e^- donor, and cyt*bo*, and cyt*bd*, which are specific for quinol as e^- donor'. What is the evidence for at least five oxidases (which are unconventionally named by Kaminski *et al.*, but will be recognizable in the context of this review)?

Cytochrome aa_3 is present, based on the finding of a *coxA* homologue and on the ability to construct a mutation in this gene (Kitts and Ludwig, 1994) associated with a clear phenotype, at least in a double mutant lacking cytochromes aa_3 and *bd* (see below). The effects of this mutation on the visible spectra are very slight. The α-band of the reduced form is weak in the wild type and its detection in the *ctaA* mutant is complicated by a rise in absorbance over a wide wavelength range between about 580 and 630 nm. Furthermore, the γ-trough at 440 nm in CO spectra, which might be expected to be largely due to the CO-ligated form of cytochrome a_3, is retained in the *ctaA* mutant. The α-region changes are attributed by Kitts and Ludwig (1994) to the presence of an alternative *a*-type cytochrome but there appears little evidence for this. In aerobic liquid cultures, cytochrome aa_3 appears to be the only oxidase in this bacterium that has actually been shown to be functional by the criterion of light-reversible CO recombination (Stam *et al.*, 1984).

Cytochrome *bd* is present based on the very distinctive absorbance spectrum in the α-region. However, its presence in the wild-type strain is hard to discern; it is clearest in a cytochrome *c*-negative mutant (Kitts and Ludwig, 1994).

The presence of cytochrome *o* is claimed (Kitts and Ludwig, 1994), but the evidence is poor: signals in the α- and β-regions of CO difference spectra are described as 'diagnostic for *o*-type cytochromes' but in fact look more like features of a low-spin haemprotein with *b*- or *c*-type haem (Wood, 1984). Signals in the γ-region near 415 nm might be from cytochrome *o*, but could also be from a CO-binding cytochrome *c*. Signals attributed to a CO-binding cytochrome *c* cannot with any confidence be claimed as arising from an oxidase. It must be emphasized that CO reactivity does not constitute a diagnostic test for oxidase activity.

Compelling molecular genetic evidence was later obtained (Mandon *et al.*, 1994) for the presence of *fixNOQP*. A *fixNO*-deleted strain retained 50% of the nitrogenase activity of the wild type in symbiosis. This is in marked contrast to

the situation in other rhizobia, where the high dioxygen affinity *fixNOQP*-encoded cytochrome *cbb'*-type oxidase is essential for nitrogen fixation *in planta* (see section 3.4). However, a double mutant lacking both cytochrome *bd* and cytochrome *cbb'* completely lacked symbiotic nitrogen fixation ability (Kaminski *et al.*, 1996). Loss of only the cytochrome *cbb'*-type oxidase was sufficient to prevent growth at 0.1% O_2. Furthermore, such a mutant was able to deplete dioxygen only down to about 3.6 μM, an activity attributed to cytochrome *bd*; submicromolar dioxygen concentrations were achieved only by strains possessing the cytochrome *cbb'*-type oxidase. It should also be noted that the K_m values for dioxygen of these oxidases have not been measured, and 'action spectra' are cited but no results presented.

In conclusion, the present experimental evidence on this diazotroph supports the presence of only three oxidases (cytochromes aa_3, *bd*, and *cbb'*), to which can be attributed certain functions. Cytochrome aa_3 appears to be important during aerobic growth (Stam *et al.*, 1984; Pronk *et al.*, 1995) but not for maintaining growth rates or nitrogen fixation, whether symbiotically or in the free-living state (Kitts and Ludwig, 1994), and indeed is markedly decreased in level in nitrogen-fixing chemostat cultures (Stam *et al.*, 1984). Cytochrome *bd* is dispensable for normal aerobic growth rates, but mutation of this oxidase gives 40% lower nitrogen fixation rates both in liquid culture and *in planta*. However, mutation of both cytochrome aa_3 *and bd* gives 70% lower nitrogen fixation *in planta*. Pleiotropic cytochrome *c* mutants, while retaining high levels of cytochrome *bd*, fixed nitrogen poorly under all conditions. This might reflect the overriding importance of cytochrome *c* oxidases, rather than quinol oxidases, for nitrogen fixation. Since during symbiosis rhizobia experience dioxygen tensions around 10 to 20 nM, what is the role of cytochrome *bd*, when it is apparently unable to deplete dioxygen tension below micromolar? Careful measurements of oxidase activity, dioxygen affinity and turnover rates will be required to solve this paradox. One possibility is that this oxidase is essential for rapid dioxygen utilization (as in *A. vinelandii*, section 4.1) to achieve a dioxygen concentration in which the very high-affinity cytochrome *cbb'* can operate. Another possibility is that the Cyd^- phenotype includes deficiencies – unrecognized at present – that lead to improper assembly or function of the very high-affinity cytochrome *cbb'*, as illustrated by several examples in section 4..

Another example of apparent redundancy in respiratory oxidases is demonstrated by the cyanobacterium *Synechocystis,* a photosynthetic prokaryote that contains complete respiratory electron transport chains on both the thylakoid and cytoplasmic membranes. The thylakoid membrane is utilized for both photosynthetic and respiratory electron transport, while the cytoplasmic membrane contains a respiratory electron transport chain but no photosynthetic reaction centres. The genome of *Synechocystis* sp. PCC 6803 contains three sets of genes for terminal respiratory oxidases (Kaneko *et al.*, 1996): the

previously identified cytochrome aa_3-type cytochrome *c* oxidase (CtaI), a second putative oxidase (CtaII) that is believed to be a cytochrome *bo′*-type quinol oxidase, and a putative cytochrome *bd* quinol oxidase (Cyd) (Howitt and Vermaas, 1998). Deletion of these respiratory oxidases had no effect on photoautotrophic or photomixotrophic growth of this organism (Howitt and Vermaas, 1998). Strains that lack one oxidase respire at near-wild-type rates, whereas those that lack both CtaI and Cyd do not respire. Thus, CtaII does not play a significant role in cellular metabolism of this microorganism under the experimental conditions tested (Howitt and Vermaas, 1998).

Not only is there redundancy in terminal respiratory oxidases in *Synechocystis*, but also in primary NADH dehydrogenases. In strain PCC6803, type 1 NAD(P)H dehydrogenase (NDH-1) is present in both the thylakoid and cytoplasmic membranes. This class of NDH is a multisubunit complex containing Fe–S clusters and FMN is proton translocating. In *Synechocystis*, NDH-1 is important for full respiratory activity and photosynthesis. Recently, three open reading frames have been identified in the *Synechocystis* genome (Kaneko *et al.*, 1996) that may encode type-2 NAD(P)H dehydrogenase (NDH-2). This class of NDH comprises a single subunit containing FAD but no Fe–S clusters. Mutations in each open reading frame (designated *ndbA*, *ndbB* and *ndbC*) led to only small changes in photoautotrophic growth rates and respiratory activities. However, in strains lacking photosystem I, mutation of one or more of the NDH-2s resulted in tolerance of much higher light intensities. These proteins may play alternative roles as redox sensors responding to the redox state of the plastoquinone pool (Howitt *et al.*, 1999).

It is now recognized that oxidase polypeptides are promiscuous with respect to the haem types that can be bound. In the cytochrome *bo′*-type oxidase of *E. coli*, the low-spin haem is generally haem B (protohaem) and the high-spin haem is of the recently described O-type. However, Puustinen *et al.* (1992) showed that cytochrome *bo′* isolated from *E. coli* strains overexpressing this oxidase possessed two types of haem complement. In some strains, 70% of the enzyme had haem O at both sites, without measurable effect on polypeptide composition and enzyme activity. Similarly, in cyanobacteria grown 'semi-anaerobically', haem A in subunit I of cytochrome aa_3 is replaced by haem O, without apparent effect on electron transfer properties. *Anabaena* and *Nostoc* species grown semianaerobically with thiosulfate and ammonium contain haem D at low levels which appears to associate with the COI subunit of cytochrome aa_3 (Peschek *et al.*, 1995) However, no ligand-binding or kinetically competent haem D-containing oxidase could be demonstrated (Fromwald *et al.*, 1999). A further example is offered by oxidases that, on the basis of sequence similarity, are unmistakably related to the *cydAB*-encoded cytochrome *bd* of *E. coli*, yet do not show any spectral signals for the characteristic haems *d* and b_{595}. These include the *cio*-encoded oxidase of *Ps. aeruginosa* (Cunningham *et al.*, 1997) and an oxidase revealed in the emerging genome sequence of *Campylobacter*

jejuni (http://www.sanger.ac.uk/projects/C-jejuni/; R.K. Poole and S. Park, unpublished). In neither case is the nature or function of the oxidase understood. The physiological consequences, which may be very subtle, of these replacements is not understood.

There is redundancy not only in the number of respiratory oxidases some bacteria possess, but also in the structure of oxidases for the generation of a Δp. For example, an alternate second oxidase in *S. acidocaldarius*, a SoxM-type, has been described with high homology to haem–Cu oxidases (Castresana *et al.*, 1995). This oxidase is a fusion between two central components of cytochrome oxidases, subunit I and subunit III. Both oxidases contribute to the generation of Δp, but by different mechanisms, and so there appears to be redundancy within this oxidase for the generation of a Δp.

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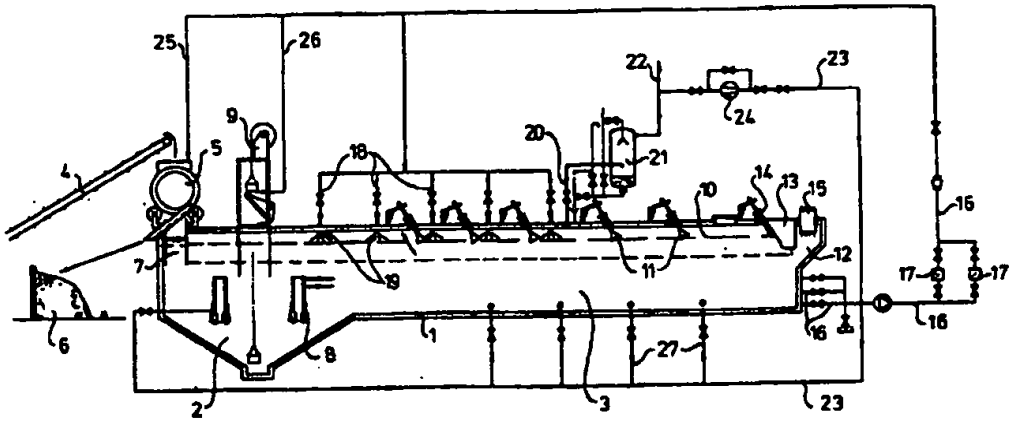
(54) Title: METHOD AND DEVICE FOR ANAEROBIC FERMENTATION OF SOLID ORGANIC WASTE SUBSTANCES



(57) Abstract

For the anaerobic fermentation of solid organic waste substances, solid waste is mixed with liquid material, particularly anaerobic slurry, and the temperature of the mixture is brought to a value between 25 and 70 °C, preferably between 30 and 40 °C (mesophilic) and between 55 and 65 °C (thermophilic). By means of spontaneous rising of lightweight material and by means of flotation, a layer (10) of solid material floating on a methane-generating zone is formed from said mixture in a reactor. Said floating layer moves from a supply end (7) or a mixing section (2) to a discharge end (14) of the reactor, hydrolysis and acidification of solidorganic material taking place in the floating layer. The fermented floating layer is discharged via the discharge (14) end independent of the residence time of fluid and slurry in the methane-generating zone under the floating layer.

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Title: Method and device for anaerobic fermentation of solid organic waste substances.

In the first instance, the invention relates to a method for the anaerobic fermentation of solid organic substances in a reactor tank in which there is a mixture of the solid organic substances and an anaerobic fluid, in which a layer of material floating on a methane-generating fluid is moved from a supply end to a discharge end of said reactor tank and a methane-forming reaction is induced in the methane-generating fluid under the floating layer, and in which fluid is sprayed in and/or on the floating layer.

Such a method is described in US-A-4334997.

Solid waste substances may consist, inter alia, of vegetable, fruit and garden waste, household waste and organic industrial waste.

Purification of sewage and the processing of manure has, for decades, employed fermentation processes. Fermentation leads to the production of biogas and to stabilization of waste or slurry. Increasingly, however, fermentation processes are also being used to process waste from the agro-industry and household waste (such as vegetable, fruit and garden waste).

When use is made of a completely mixed reactor or a plug-flow reactor, the residence times of the waste to be fermented and of the biomass (methane-generating sludge) are identical to each other. However, the growth rate of methane-generating bacteria is relatively low, which results in the residence time of the biomass and thus of the material to be fermented having to be relatively long (20 to 30 days). This results in relatively long reactor tanks. Although systems are known in which a separation is brought about between the residence times of fermenting material and methane-generating biomass, they usually make use of a plurality of reactors with complicated separation systems between them. This also leads to high production and operating costs.

In the method according to the abovementioned US patent specification, the floating layer is moved and discharged independent of the methane-generating fluid zone. In other words, the residence times of the floating layer and the methane-generating slurry do not have to be identical to each other. The fluid sprayed on the floating layer consists of deoxygenated water which fulfils only a transport function for the floating layer. The fluid will have to be vigorously squirted onto and in the floating layer, which will also have a mixing effect and, as a result

of this, solid portions of the floating layer are squirted into the methane-generating fluid, to the detriment of the thickness of the floating layer. In each case, no attempt is made to ferment the components of the floating layer. The floating layer is regarded only as an inconvenience and is therefore kept as thin as possible. The floating layer is discharged from the reactor tank as quickly as possible.

The object of the invention is to generate a controlled fermentation reaction in the floating layer.

To this end, the method mentioned in the preamble is characterized in that the fluid sprayed in and/or on the floating layer is extracted from the methane-generating zone under the floating layer in order to induce fermentation in the floating layer and also, by means of percolation, to remove acid fermentation products from the floating layer and to drive them to the methane-generating zone under the floating layer, and in that the floating layer is moved in such a controlled manner that the said fermentation reaction can take place in the floating layer.

Solid waste and anaerobic slurry could be mixed outside the reactor, but it is preferable for this mixing to be carried out in a mixing section of the actual reactor.

During mixing, heavy material which has sunk has to be removed periodically.

The anaerobic slurry will have to be brought to the desired temperature in order to achieve adequate fermentation performance levels. In the case of the mesophilic bacteria, this means that the temperature has to be brought to between approximately 30 and approximately 40°C, whilst in the case of thermophilic bacteria, a temperature of between approximately 55 and approximately 65°C is favourable.

According to the invention, by means of spontaneous floatation of the light solid material and sinking of the biomass, a separation is brought about between said two materials, and both materials are fermented adequately independently and in their own time. It is essential that fluid from the methane-generating zone is sprayed in and/or on the floating layer, by means of which fermentation in the floating layer is achieved.

The biogas formed in the methane-generating zone flows upwards and bubbles through the floating layer, as a result of which, on the one hand, the floatation is enhanced and, on the other hand, mixing and breaking-open of the floating layer take place. Mixing can be intensified

if the fluid withdrawn from the methane-generating zone is also used to improve the intake of solid material and mixing.

The 1- to 2-metre-thick floating layer will be broken up to a certain degree and the contents of the reactor will be better mixed if the biogas formed is at least partially recirculated by being injected, at different locations, into the lower portion of the reactor.

The residence time of the fermenting material in the floating layer in the reactor is approximately 5 days. During this time, the floating layer is moved towards the discharge end of the reactor using mechanical means.

At the discharge end of the reactor, the floating layer is pushed under a baffle forming part of a water seal or is removed via another mechanism. The baffle can be adjusted in order to regulate the thickness of the floating layer.

The invention also relates to a reactor for implementing the above method, comprising a reactor tank with a supply end for a mixture of solid waste and anaerobic fluid and a discharge end for a layer floating on the methane-generating fluid, means for moving the floating layer from the supply end to the discharge end, and means for spraying fluid in and/or the floating layer and means for discharging the floating layer out of the reactor, via a water seal, at the discharge end independent of the fluid and slurry located under the floating layer.

Such a reactor is also known from said US-A-4334997.

In order to be able to implement the method according to the invention, the means for spraying fluid in and/or on the floating layer are connected to lines which can extract fluid from the methane-generating zone under the floating layer.

In this case, means may be present for discharging the biogas formed in the reactor which are installed at different locations at the bottom of the reactor.

The means for moving the floating layer may consist of a hydraulically movable blade which can hinge in the forward direction of the floating layer, and can execute a downward translational movement in the floating layer during said hinging movement, and, when it has reached an approximately vertical position can move upwards to a position outside the floating layer and, finally, can hinge back to the starting position. A blade could also be moved mechanically to follow a parallelogram-shaped path: in sequence, obliquely forwards, in the direction of the discharge end of the reactor, obliquely back upwards and, finally, in the direction

of the supply end of the reactor.

The invention will now be described in greater detail on the basis of the figure which gives a diagrammatic illustration of the reactor according to the invention.

The figure shows a reactor tank 1 which is closed at the top, consisting of a mixing part 2 and a fermentation part 3.

A supply conveyor 4 for solid organic waste, such as vegetable, fruit and garden waste, opens out, via its discharge end, above a rotating drum sieve 5 which separates the very coarse material, such as branches, car tyres and concrete blocks from the waste for fermentation formed by the material which passes through the sieve. The very coarse waste falls onto a storage area 6 and the material passing through the sieve falls via an inlet 7, designed like a water seal, into the mixing part 2. In said part, stone, glass, ceramic material, metal and coarse sand sink to the bottom and the newly arrived components to be fermented and methane-generating biomass in the reactor are mixed. Then, in the mixing part, the mixture is heated to a temperature of between 25 and 70°C, preferably between 30 and 40°C (mesophilic) or between 55 and 65°C (thermophilic) by means of heating/mixing units 8. The latter ones consist of double-walled vertical nozzles in whose cavity hot water flows. From time to time, the material which has sunk and is lying on the bottom of the mixing part is removed by means of a grab-crane 9.

In the fermentation portion 3, the actual biological conversions take place, as a result of which, owing to the rising up of the fibrous material and flotation, caused by rising bubbles of biogas, a floating layer 10 is formed with a thickness of 1 to 2 metres. Said layer is pushed by means of hydraulically driven blades 11 in the direction of the discharge end 14 of the reactor, which discharge end is designed as a water seal. Before said discharge end there is a baffle 13 and a plunger 12 for pushing the fermented material of the floating layer 10 under the baffle 13 through towards the water seal 14, which allows the material to fall onto a discharge area. The blades 11 can hinge in the forward direction of the floating layer and, simultaneously, describe a downward translational movement. When said blades have reached a vertical position, they move upwards and hinge towards the starting position.

During the approximately 5-day-long transportation of the material in the floating layer, from the intake 7 to the outlet 12, organic material is hydrolysed and acidified, while methane is also formed in the floating layer. At a pH of between 6 and 7, solid

substances, such as starch and protein, are biologically converted into dissolved substances such as sugar, acetic acid and amino acids. The acidification products must not accumulate because, in the event of a highly acidified floating layer, the biological decomposition process will be halted. Discharge of acidification products from the floating layer towards the methane-generating zone underneath takes place by percolation through the floating layer of buffer fluid which is drawn off by means of lines 16 and sieves 17 from the methane-generating zone and is returned via lines 18 and spraying heads 19 in and/or on the floating layer. The drawn-off fluid also contributes to fermentation in the floating layer.

The dissolved acidification products are converted into biogas in the methane-generating biomass under the floating layer 10. Said biogas flows upwards and leaves the reactor via the line 20 after it has bubbled through the floating layer, which gives rise to extra mixing and opening-up of the floating layer. As a result of this, there will be no formation of a crust, blind spots or dead spaces.

The biogas flows via the line 20 to a tank 21 in which foam is separated off. From there, the gas flows via a discharge line 22 towards, for example, a generator. A branch line 23 of the line 22 carries a portion of the biogas, after the latter has passed through a compressor 24, to a number of lines 27 opening out in the lowermost portion of the reactor. Said extra biogas intensifies the mixing in the methane-generating zone and causes greater opening-up of the floating layer.

The fluid drawn off from the methane-generating zone via the line 16 can, in addition to being used for said percolation through the floating layer, be used for improving the supply and mixing of the fermented material (see line 25) and for spraying the material which has been collected by the crane 9 (see line 26).

The degree of acidity in the floating layer will be between 6 and 7 and that in the methane-generating slurry between 7 and 8.

The most important advantage of the reactor described and the method described is that the residence times of the floating layer and methane-generating zone may be different without complicated apparatus. The residence time of the material in the floating layer is approximately 5 days and that in the methane-generating zone, depending on the dry-matter content (which is usually less than 7%) 20 days, for example. Furthermore, the fermentation in the floating layer itself is enhanced.

The fermented material discharged can, for example, be

processed into compost.

The slurry discharged from the methane-generating zone can be separated out in a centrifuge, resulting in water on the one hand and a solid substance and methane bacteria on the other.

Various modifications and additions are possible within the scope of the invention.

CLAIMS

1. Method for the anaerobic fermentation of solid organic substances in a reactor tank in which there is a mixture of the solid organic substances and an anaerobic fluid, in which a layer of material floating on a methane-generating fluid is moved from a supply end to a discharge end of said reactor tank and a methane-forming reaction is induced in the methane-generating fluid under the floating layer, and in which fluid is sprayed in and/or on the floating layer, characterized in that the fluid sprayed in and/or on the floating layer is extracted from the methane-generating zone under the floating layer in order to induce fermentation in the floating layer and also, by means of percolation, to remove acid fermentation products from the floating layer and to drive them to the methane-generating zone under the floating layer, and in that the floating layer is moved in such a controlled manner that the said fermentation reaction can take place in the floating layer.

2. Method according to Claim 1, characterized in that mixing of the solid waste and anaerobic slurry takes place in a mixing section of the reactor.

3. Method according to Claim 1 or 2, characterized in that, during mixing, heavy material which has sunk is periodically removed.

4. Method according to one of the preceding claims, characterized in that fluid from the methane-generating zone is also used to improve the intake of solid material and mixing.

5. Method according to one of the preceding claims, characterized in that said biogas is at least partially recirculated by being injected, at different locations, into the lower portion of the reactor.

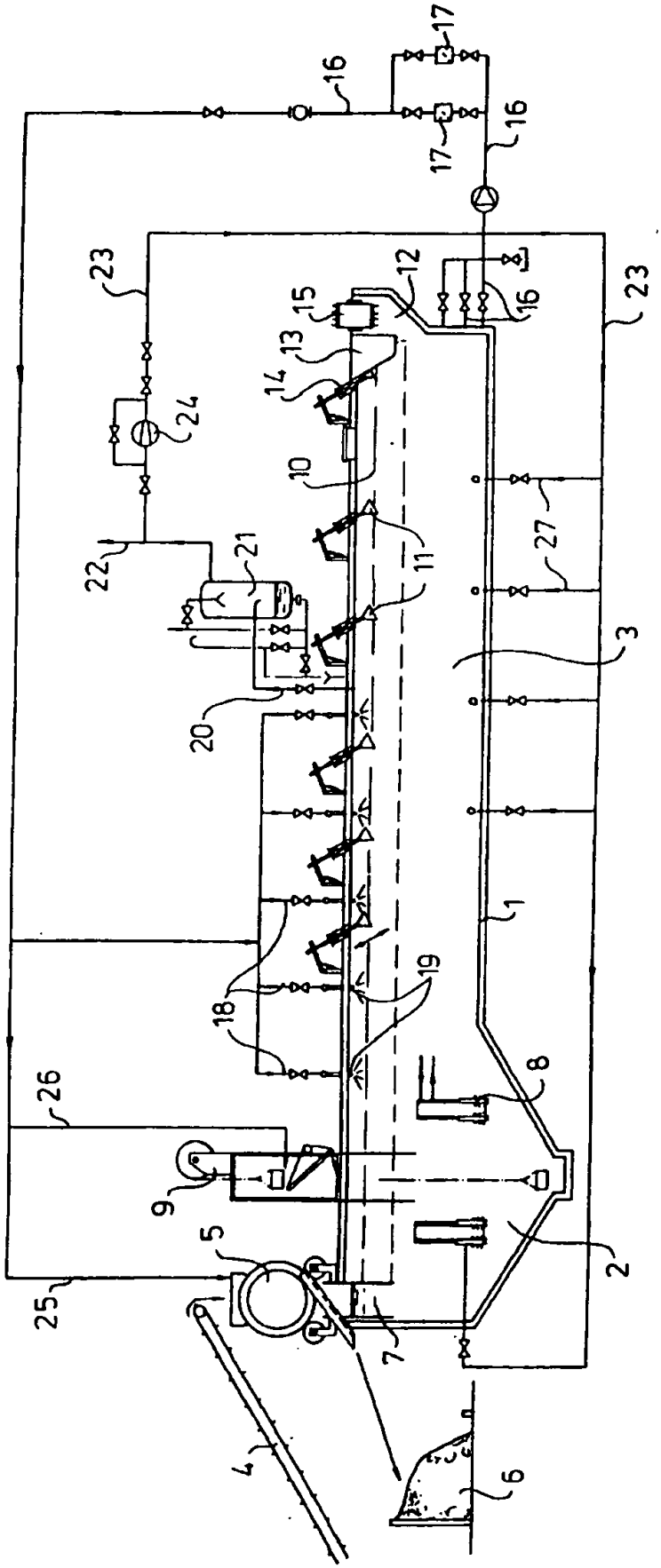
6. Method according to one of the preceding claims, characterized in that the floating layer is moved to the discharge end of the reactor using mechanical means.

7. Reactor for implementing the method according to one of the preceding claims, comprising a reactor tank with a supply end for a mixture of solid waste and anaerobic fluid and a discharge end for a layer floating on the methane-generating fluid, means for moving the floating layer from the supply end to the discharge end, and means for spraying fluid in and/or on the floating layer and means for discharging the floating layer out of the reactor, via a water seal, at the discharge end independent of the fluid and slurry located under the floating layer, characterized in that the means for spraying fluid in and/or on the floating layer are connected to leads which can withdraw the fluid from

the methane-generating zone under the floating layer.

8. Reactor according to Claim 7, characterized by means for discharging the biogas formed in the reactor which are installed at different locations in the bottom of the reactor.

9. Reactor according to Claim 7 or 8, characterized by mechanical means for moving a floating layer formed in the reactor in a controlled manner to the discharge end of the reactor.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INTERNATIONAL SEARCH REPORT

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A,4 334 997 (R. E. PETERSON) 15 June 1982 cited in the application see column 2, line 53 - column 4, line 64; claims	1-5,8
Y	EP,A,0 048 675 (SOCIETE ENTREPRISE METALLURGIQUE D'ARMOR) 31 March 1982 see claims; figure 1	1-5,8
Y	GB,A,2 204 056 (STIG ARVID HENDRIKSON) 2 November 1988 see page 4, line 12 - line 27; figures 1,5	1,4,5,8
A	BE,A,888 670 (EKSELSE KONSTRUKTIE WERKEN) 28 August 1981 see page 3, paragraph 2; figure	5,8

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15

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference UA-338 PCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/26950	International filing date *(day/month/year)* 16 NOVEMBER 1999	Priority date *(day/month/year)* 18 NOVEMBER 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE UNIVERSITY OF AKRON		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21 APRIL 2000	31 OCTOBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorthea Lawrence for PADMA BASKAR Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/26950

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages 1-22, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the claims:
pages 23-33, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the drawings:
pages 1-6, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/26950

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-71	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-71	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-71	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-71 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a process for the production of biological materials by simultaneous aerobic and anaerobic respiration. This process includes aerating the culture medium with oxygen and supplying the culture medium with an alternative oxidant source, other than oxygen so that when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for anaerobic cellular respiration. The alternative oxidant sources are very soluble in aqueous media and can be utilized by microorganisms for cellular respiration This solves the problems with foaming which are associated with the vigorous agitation. Furthermore, the present invention utilizes an external oxidant source, such as nitrates, as the terminal electron acceptor.

---------- NEW CITATIONS ----------

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C12P 1/00, 39/00, 19/02. 19/44; C12N 1/20 and US Cl.: 435/41, 42, 74, 105, 253.3; 536/4.4

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Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PRODUCTION OF BIOLOGICAL MATERIALS BY SIMULTANEOUS AEROBIC AND ANAEROBIC RESPIRATION

(57) Abstract

A process for the production of biological products by microorganisms comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product. The invention also provides a process for increasing concentration of microorganisms in a defined culture medium.

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PRODUCTION OF BIOLOGICAL MATERIALS BY SIMULTANEOUS AEROBIC AND ANAEROBIC RESPIRATION

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority from United States Provisional Patent Application No. 60/108,837, filed on November 18, 1998.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the production of biological materials by microorganisms. The present invention more particularly relates to a process for the preparation of biological products, such as biosurfactants, by microorganisms under simultaneous aerobic and anaerobic respiring conditions.

BACKGROUND OF THE INVENTION

Cells are the real workers in biological processes. To increase process productivity, it is desirable to grow the cells to concentrations as high as possible. For aerobic biological processes, the cell concentrations employable are most commonly limited by the rate of oxygen transfer to the cell population that is achievable by a particular process. Therefore, the productivity of biological materials by aerobic fermentation processes is directly limited by the oxygen supply to the cells.

It is well known that oxygen gas is only slightly soluble in aqueous media and, therefore the supply of oxygen must be replenished by inefficient mass transfer across the gas/liquid interface. This is traditionally achieved by vigorous aeration and/or

agitation to promote the interfacial transfer of oxygen from gas bubbles to the aqueous media.

However, this limitation is especially serious in biological production processes that prevent the use of vigorous agitation or aeration to promote the interfacial transfer of oxygen gas in the aqueous media, such as in the production of highly foaming biosurfactants such as rhamnolipids, highly viscous biopolymers such as xanthan gum, oxygen sensitive products, and production of biological materials, such as antibiotics, by shear-sensitive organisms.

It is widely known in the art to produce biosurfactants, such as rhamnolipids, by conventional aerobic fermentation processes. For example, United States Patent No. 5,501,966 to Giani et al discloses a method for the biotechnological preparation of L-rhamnose by microorganisms, such as *Pseudomonas aeruginosa*. The bacterial strain *Pseudomonas aeruginosa* is used to secrete rhamnolipids, under aerobic fermentation conditions, into the culture supernatant. The *Pseudomonas aeruginosa* is fermented in a medium containing vegetable oils, such as olive, corn and sunflower oil, as the carbon source. Aeration is employed using sterile air to provide oxygen to the fermentation solution. The reference discloses that it is necessary to add a suitable anti-foaming agent to the fermentation solution during the fermentation process. The L-rhamnose is recovered directly from the culture solution by hydrolysis of the rhamnolipids, without separation of the cell material and without isolation of the rhamnolipids before hydrolysis.

Furthermore, United States Patent No. 4,628,030 to Kaeppeli et al discloses a method for the production of rhamnolipids by the microorganism *Pseudomonas aeruginosa*. According to the reference, rhamnolipids are produced by the cultivation of rhamnolipid producing microorganisms of the genus *Pseudomonas* in an aqueous

culture medium suitable for the growth of the microorganism. The microorganism is cultured in a continuous submerged culture under aerobic conditions and with a continuous supply of fresh culture medium, and continuous removal of a solution of partially spent culture medium and produced rhamnolipids; and limiting the amount of at least two essential growth substances selected from the group consisting of carbon, nitrogen, sulfur, phosphorous, sodium, potassium, magnesium, calcium, iron, zinc, manganese, boron, cobalt, copper and molybdenum, in the culture medium such that the quantity of essential growth substance in the partially spent culture medium is less than half of the amount in the fresh culture medium.

United States Patent No. 4,814,272 to Wagner et al discloses a method for the microbiological production of rhamnolipids comprising culturing the microorganism *Pseudomonas* species 2874 under aerobic conditions in an aqueous nutrient solution containing at least one carbon source at a pH value of 6.5 to 7.3 and a temperature of 30° to 37°C. The aqueous culture is either extracted directly with a suitable solvent and evaporated, or the resulting wet cell mass is separated from the culture broth and incubated with a carbon source to further increase rhamnolipid production.

United States Patent No. 4,933,281 to Daniels et al discloses a method for producing rhamnose comprising the steps of growing the microorganism *Pseudomonas* in a defined culture medium containing vegetable oil to produce rhamnolipids; hydrolyzing the rhamnolipids to form rhamnose and 3-hydroxydecanoic acid; and separating the rhamnose from the acid. During fermentation, sterile air is sparged into the fermentor at a rate of 0.1 to 1.0 VVM (volume air per volume fermentor liquid per minute), with a rate of 0.5 VVM being most perferred.

In addition to biosurfactant production, it is also known to produce viscous biopolymers, such as xanthan gum, by conventional aerobic fermentation processes.

United States Patent No. 4,352,882 to Maury discloses a method for production of a polysaccharide gum, such as xanthan gum, by microemulsion comprising inoculating an aqueous culture medium comprising a carbohydrate source and a nitrogen source with a polysaccharide gum-producing microorganism, mechanically agitating and aerating the culture medium under aerobic conditions to effect fermentation thereof, wherein the culture medium is dispersed in about 20 to 80% of its weight of a water insoluble oil in which the resultant polysaccharide is also insoluble. The reference further teaches that the oil in the microemulsion significantly increases the oxygen transfer efficiency leading to an increased rate of reaction.

The above referenced conventional methods of biological production of biosurfactants, including rhamnolipids, have serious disadvantages. The most significant technical problem associated with the above referenced methods of rhamnolipid production under aerobic conditions is the extensive formation of foam. Due to the rapid foam formation and high foam stability, the elimination of foam during biological processes continues to be a problem.

There have been numerous attempts to utilize chemical anti-foam agents to eliminate foam formation during aerobic production of biosurfactants. However, the known anti-foam agents are very expensive, and may affect cell metabolism, downstream product recovery and purification, and wastewater processing.

There have also been attempts to control the foaming associated with aerobic fermentation in biological processes through the use of a mechanical apparatus that is in fluid communication with the fermentation tank. For example, United States Patent No. 5,476,573 to Hirose et al. discloses an apparatus for defoaming and controlling aerobic culture fermentation comprising a first means for separating vapor from liquid of a foam; a second means for separating residual liquid of said vapor received from

said first means for separating, in fluid communication with said first means for separating; a means for recirculating liquid from said first means for separating and condensed residual liquid from said second means for separating, said means for recirculating being in fluid communication with said first means for separating and said second means for separating, and a sensor for detecting foams, located between and in fluid communication with said first means for separating and said second means for separating. The reference further discloses that an optional defoaming device may be included, which may be based on either a rotary body rotating at a high speed by use of an electric motor which beat the foams, or on a centrifugal atomizer.

It is, therefore, desirable to develop a process for production of biological materials to avoid the problems associated with oxygen limitation and foam formation arising from continuous aeration and vigorous agitation that is required during known biological production processes.

In addition, with the technological advancements in the area of genetic engineering of cells, it is desirable to develop a process for the production of biological materials, that can employ genetically engineered or manipulated host cells, which avoids the problems associated with oxygen limitation and foam formation arising from continuous aeration and vigorous agitation that is required during known biological production processes.

Heretofore, the prior art has not taught to produce biological materials, such as biosurfactants, viscous biopolymers, oxygen sensitive products, and the like by simultaneous aerobic and anaerobic respiration processes.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for the production of biological materials that eliminates the problems associated with oxygen limitation encountered in solely aerobic bioprocesses.

It is another object of the present invention to provide a method for production of biological materials that eliminates foam formation problems associated with solely aerobic production of biological materials.

It is another object of the present invention to provide a method for production of biological materials that allows use of high cell concentrations.

It is another object of the present invention to provide a method for production of biological materials that increases volumetric productivity of biological products.

It is another object of the present invention to provide a method for production of biological materials that reduces the cost of downstream recovery and purification of the final biological products.

It is another object of the present invention to provide a method to increase production of biological materials that are oxygen sensitive.

It is another object of the present invention to provide a method to produce biological materials that can utilize or employ genetically engineered or manipulated microorganisms or cells.

The foregoing objects, together with the advantages thereof over the known art relating to aerobic production of biological materials, which shall become apparent from the specification which follows, are accomplished by the invention as hereinafter described and claimed.

The present invention, therefore, provides a process for the production of biological products by microorganisms comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the cellular respiration requirements of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms will utilize the available oxygen within the medium and another portion of the microorganisms within the culture medium will simultaneously utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

The present invention also provides a process for the preparation of biological products under anaerobic respiring conditions comprising: selecting a microorganism that is capable of utilizing an alternative oxidant source other than oxygen for cellular

respiration under anaerobic conditions; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; supplying an alternative oxidant source other than oxygen to the culture medium; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

The present invention also provides a process for creating an increased concentration of microorganisms in a defined medium comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms will utilize the oxygen available within the medium and another portion of the microorganisms within the culture medium will simultaneously utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired concentration of microorganisms within the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the growth of *Pseudomonas aeruginosa* on various carbon substrates under anaerobic denitrification conditions.

Figure 2 is a graph showing the growth of *Pseudomonas aeruginosa* over time in aqueous culture media containing an initial addition of rhamnolipids as compared to growth in an aqueous medium without rhamnolipids. Cell growth is measured by the increase in cell protein (g/L).

Figure 3 is a graph showing *Pseudomonas aeruginosa* growth on a glycerol substrate under anaerobic denitrifying conditions.

Figure 4 is a graph showing *Pseudamonas aeruginosa* growth on a palmitic acid substrate under anaerobic denitrifying conditions.

Figure 5 is a graph showing *Pseudomonas aeruginosa* growth on a stearic acid substrate under anaerobic denitrifying conditions.

Figure 6 is a graph showing rhamnolipid production by *P. aeruginosa* under anaerobic denitrifying conditions followed by fermentation under aerobic conditions, using palmitic acid as the carbon source.

DETAILED DESCRIPTION OF THE INVENTION

It is now been discovered that a method utilizing both aerobic and anaerobic respiration can be used to produce high cell concentrations within a culture medium, which results in an increase in the volumetric productivity of biological products, such

as surfactants and viscous biopolymers. It is envisioned that the method of the present invention can be useful as a batch or continuous process for the production of biological materials. The process of the present invention is especially useful in the production of biosurfactants, such as rhamnolipids. Rhamnolipids are anionic extracellular biosurfactants that are useful in many commercial applications in the petroleum, pharmaceutical and food processing industries. The present invention is premised on the fact that various species of microorganisms can use certain alternative oxidants, such as nitrates and the like, other than molecular oxygen for purposes of metabolic or cellular respiration to avoid problems associated with oxygen limitation in bioprocesses. By using alternative oxidant sources, the serious limitations associated with oxygen supply to the cells, such as reduced cell number and foam generation can be eliminated.

The present invention provides a process for the production of biological products by microorganisms. A suitable microorganism that is capable of undergoing or utilizing anaerobic respiration must be selected. A defined culture medium must be provided that is suitable for the growth of the microorganism to carry out the biological processes. The medium comprises at least one carbon source for the microorganism. Once a suitable culture medium has been selected, a desired cellular concentration or quantity of microorganism is introduced or added to the culture medium. The culture medium is aerated with oxygen and also supplied with an alternative oxidant source.

It should be noted that the process has a maximum oxygen transfer or supply rate into the culture medium. When the cellular respiration requirements (the oxygen requirements) of the microorganisms within the culture medium is less than the maximum rate of oxygen transfer or supply into the culture medium, then the microorganisms will utilize the oxygen within the culture medium for cellular respiration. However, as the concentration of cells within the medium begins to

increase, the cellular respiration requirements, and consequently the oxygen consumption, of the microorganisms within the culture medium increases. When the cellular respiration requirements of the microorganisms within the culture medium becomes greater than the maximum oxygen transfer or supply rate into the culture medium, a portion of the microorganisms will utilize the available oxygen within the medium, and another portion of the microorganisms within the medium will simultaneously begin to utilize the alternative oxidant source for cellular respiration requirements. This process, therefore, enables the microorganisms to reach high concentrations within the medium and remain viable throughout productions of biological materials. With a portion of the microorganisms using the alternative oxidant source for anaerobic respiration, cell growth continues and the concentration of cells within the medium reaches concentrations that would otherwise be impossible due to oxygen limitation.

The culture medium is maintained at a desired pH and temperature, and the culture medium is allowed to incubate for a time sufficient to produce a desired quantity of a biological product. The resulting biological product is isolated and recovered from the culture medium.

The microorganisms that are useful in the present invention are those selected from bacteria, yeast, molds, archaea, and the like. Preferred microorganisms are facultative aerobic bacteria and obligate anaerobic bacteria.

Facultative aerobic bacteria are those species of bacteria that can either utilize oxygen for respiration purposes under aerobic conditions, or can utilize alternative oxidants other than oxygen for respiration purposes in the absence of oxygen. Suitable species of facultative aerobic bacteria that may be used in the present invention include, but are not limited to, nitrate/nitrite respiration bacteria such as *Pseudomonas*

aeruginosa, Pseudomonas fluorescens, Paracoccus denitrificans, Micrococcus halodenitrificans, Klebsiella aerogenes, Escherichia coli, and the like; hyperthermophilic Archaea bacteria such as *Acidianus*; and the fumarate respiration bacteria such as *Wolinella succinogenes, Desulfovibrio gigas, Clostridia, Escherichia coli* and *Proteus rettgeri*. A preferred facultative aerobic bacterium is that of the genus *Pseudomonas*.

According to the present invention the quantity or concentration of the microorganism that is added to the culture medium for processes employing growing cells is preferably from about 0.1 g/L to about 10 g/L, more preferably from about 0.5 g/L to about 5 g/L. For processes employing non-growing (stationary phase) cells, the quantity or concentration of the microorganism that is added to the culture medium is preferably from about 5 g/L to about 50 g/L.

The production of biological products according to the method of the present invention requires that a defined liquid culture medium be selected. The liquid culture medium contains at least one carbon source (substrate) for production of biological products. The liquid culture medium used in the present invention can be any known culture medium that comprises nutrients that can support cellular growth, particularly microbial growth. Without limiting the processes of the present invention to any particular culture medium, a representative liquid culture medium formulation may comprise the following mineral substituents: 4 g/liter of NH_4Cl, 11 g/liter of K_2HPO_4, 0.5 g/liter NaCl, 0.3 g/liter $MgSO_4 \cdot 7H_2O$, 0.03 g/liter $FeCl_3 \cdot 6H_2O$, 0.01 g/liter $CaCl_2$, and 0.01 g/liter $MnCl_2 \cdot 4H_2O$.

Carbon sources useful in the present invention include, but are not limited to, fatty acids; glycerol; low molecular weight organic acids such as malate, acetate,

pyruvate and the like; vegetable oil; yeast extract; peptone; and carbohydrates such as glucose.

Suitable fatty acids that can be utilized in the present invention include, but are not limited to, fatty acids such as palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, linolenic acid, and the like. Palmitic acid is a preferred fatty acid that may be utilized in the processes of the present invention.

Vegetable oils, such as corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil, sunflower oil, and the like, may be used as the carbon substrate in the present invention. A preferred vegetable oil for use in the present invention is corn oil.

As described hereinabove, the culture medium is simultaneously aerated with oxygen and supplied with an alternative oxidant source. The population of microorganisms in the culture medium that are not utilizing oxygen for cellular respiration will utilize an alternative oxidant present in the medium instead of oxygen as the final electron acceptor in the cellular respiratory chain. The term "oxidant", as used throughout the specification, refers to the molecules or compounds that can serve as the terminal electron acceptor in the respiratory chain of a cell. According to the present invention, suitable alternative oxidants are selected from nitrates, nitrites, sulfates, sulfites, carbon dioxide or carbonates, bicarbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

According to the present invention suitable nitrates include, but are not limited to, those nitrates selected from sodium nitrate, potassium nitrate, calcium nitrate,

magnesium nitrate, ammonium nitrate, nitric acid, and the like. A preferred nitrate for use as the alternative oxidant source is sodium nitrate.

According to the present invention suitable nitrites include, but are not limited to, those nitrites selected from sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrites, nitrous acid and the like. A preferred nitrite for use as the alternative oxidant source is sodium nitrite.

According to the present invention suitable sulfates include, but are not limited to, those sulfates that are selected from sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, dilute sulfuric acid, and the like.

According to the present invention suitable sulfites include, but are not limited to, those sulfites that are selected from calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite, manganese sulfite, and the like.

According to the present invention suitable carbonates and bicarbonates include, but are not limited to, those carbonates and bicarbonates that are selected from calcium carbonate, sodium carbonate, potassium carbonate, calcium bicarbonate, sodium bicarbonate, potassium bicarbonate, carboxylic acid, and the like.

Suitable fumarates useful in the present invention include, but are not limited to, those fumarates that are selected from the group consisting of disodium fumarate ($C_4H_2O_4Na_2$), sodium fumarate ($C_4H_3O_4Na$), dipotassium fumarate ($C_4H_2O_4K_2$), potassium fumarate ($C_4H_3O_4K$), fumaric acid ($C_4H_4O_4$), and the like.

Depending on the microorganism employed, the alternative oxidant is maintained in the culture medium at a concentration from about 0.01 g/L to about 10 g/L preferably at a concentration from about 0.05 g/L to about 5 g/L, and more preferably from about 0.1 g/L to about 0.5 g/L.

According to the present invention, a desired amount of a surfactant may also be added to the culture medium to facilitate the mass transfer of the otherwise poorly soluble or insoluble carbon substrate into the culture medium. The addition of the surfactant to the culture medium facilitates the dispersion or solubilization of the carbon source into the culture medium. Furthermore, the addition of the surfactant to the culture medium assists in the penetration of the carbon source through the cell wall of the microorganism. The amount of surfactant to be added to the culture medium is from about 0.01 g/L to about 10 g/L, preferably from about 0.05 g/L to about 1 g/L, and most preferably from about 0.1 g/L to about 0.5 g/L.

The microorganism is incubated with the carbon substrate contained in the culture medium for a time sufficient at a desired temperature and pH to produce a desired quantity of a biological product. The temperature of the culture medium influences the growth and the survival of the microorganism employed. For every organism there is minimum temperature below which growth no longer occurs, an optimum temperature at which growth is most rapid, and a maximum temperature above which growth falls sharply to zero. Many microorganisms have temperature ranges as low as about 5°C to about 10°C, while some microorganisms have optimum temperatures greater than about 100°C. According to their temperature optima, microorganisms are classified into psychrophiles, having temperature optima of less than about 10°C, mesophiles, having temperature optima from about 15°C to about 45°C, thermophiles, having temperature optima of greater than about 45°C, and hyperthermophiles having temperature optima of greater than about 80°C. Therefore,

the temperature of the culture medium throughout the incubation period is dependant on the microorganism selected. For example, the temperature of the culture medium for *P. aeruginosa* during incubation is preferably carried out in a temperature range of about 20°C to about 40 °C, more preferably in a temperature range of about 27°C to about 38 °C, and most preferably between about 30°C and about 37°C.

Throughout the incubation period, the pH of the culture medium is maintained in an optimal pH range, which is dependent on the species of microorganism chosen.

In another embodiment, the present invention provides a process for the production of biosurfactants, such as rhamnolipids, by the facultative aerobic bacterium, *Pseudomonas aeruginosa*. In the absence of oxygen, *Pseudomonas aeruginosa* within the culture medium will utilize an alternative oxidant source, such as sodium nitrate, for cellular respiration purposes. It has been found that limiting the essential growth nutrient phosphorous from the culture media brings about the onset of the stationary phase, and facilitates increased rhamnolipid production by *Pseudomonas aeruginosa*.

The temperature range for the production of rhamnolipids by *Pseudomonas aeruginosa* by anaerobic denitrification is from about 20 to about 40°C, more preferably from about 27 to about 38°C, and most preferably from about 30 to about 37°C. The pH range of the culture medium for the production of biosurfactants by *Pseudomonas aeruginosa* is optimally from about 6 to about 7, more preferably between about 6.5 to about 6.8.

It should be noted that the process of the present invention can utilize genetically engineered or manipulated host microorganisms or cells for production of biological materials, provided that the genetically engineered host cell is capable

utilizing an alternative oxidant source. The process, including the selection of a suitable culture medium, carbon substrate, alternative oxidants and reaction conditions, is essentially the same as disclosed hereinabove, but employs genetically engineered microorganism. A DNA sequence encoding for a desired biological product is selected. A suitable host microorganism that is capable of undergoing anaerobic respiration is transfected with the DNA sequence, and is added to a suitable culture medium.

In another embodiment of the present invention, production of biological products under anaerobic conditions is provided. A culture medium suitable for the growth of said microorganism, and comprising at least one carbon source is provided. The culture medium is inoculated with a desired cellular concentration of the microorganism. An oxidant other than oxygen is supplied to the culture medium, under anaerobic conditions and in the absence of oxygen. The culture medium is maintained at a desired pH and temperature, and allowed to incubate for a time sufficient to produce a desired quantity of a biological product. An essential cellular growth nutrient may be substantially limited from the culture medium to inhibit cell growth and facilitate to the production of biological product.

In addition to the facultative aerobes described hereinabove, obligate anaerobes can be employed as the microorganism in this embodiment. Preferred obligate anaerobes are obligate anaerobic bacteria.

Obligate anaerobic bacteria are those species of bacteria that can only survive and grow under anaerobic conditions, that is, in the complete absence of oxygen. Suitable species of obligate anaerobic bacteria that may be used in the present invention include, but are not limited to, the homoacetogenic and methoanogenic Archaea bacteria capable of carbon dioxide/carbonate respiration; the sulfate-respiration bacteria

such as *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfobulbus, Desulfococcus, Desulfobacter, Desulfosarcine, Desulfonema*, and the like; the sulfur-respiration bacteria such as *Desulfurmonas*; hyperthermophilic Archaea bacteria, such as *Thermoproteus, Pyrococcus, Thermococcus*, and the like; and ferric ion (Fe^{3+}) respiration bacteria such as *Shewanella putrefaciens*.

An essential growth nutrient may be limited from the culture medium in order to regulate cellular growth and to reach the resting or stationary phase. An essential growth nutrient that can be excluded or removed from the liquid culture media is selected from sulfur, phosphorous, nitrogen, magnesium, calcium and iron. The terms "resting phase" and "stationary phase", as used throughout the specification, refer to the phases when the cells are not undergoing cellular division.

Examples of biological products that can be produced according to the methods of the present invention, but are not limited to, biosurfactants, viscous biopolymers, proteins, enzymes, specialty chemicals, oxygen sensitive products, and products produced by shear sensitive microorganisms.

The biosurfactants that can be produced according to the methods of the present invention include, but are not limited to, rhamnolipids, sophorolipids, trehalose mycolates, trehalose esters, monosaccharide mycolates, disaccharide mycolates, trisaccharide mycolates, phospholipids, fatty acids, gramicidens, polymyxins, omithine-lipid, cerilipin, lysin-lipid, surfactin, subtilisin, peptide-lipid, heteropolysaccharide, lipoheteropolysaccharide, poly-saccharide-protein, manno-protein, carbohydrate-protein, mannan-lipid complex, mannose/erythrose-lipid, carbohydrate-protein-lipid-complex and fimbriae.

The viscous biopolymers that can be produced according to the methods of the present invention include, but are not limited to, xantham gum, pullulan, dextran and polyalginic acid.

The products produced according to the process of the present invention by shear sensitive microorganisms are selected from the group consisting of antibiotics, enzymes, cellulases, amylase, proteases, liginases, and organic acids.

GENERAL EXPERIMENTAL

The following example of rhamnolipid production by *P. aeruginosa* is set forth to illustrate the methods of the present invention. However, the examples should not be construed as limiting the present invention in any manner.

The production of rhamnolipid biosurfactants under phosphorous-limited denitrifying anaerobic conditions was evaluated. The experiment was conducted in a 2 liter Erlenmeyer flask, having a 600 milliliter working volume. The experiments were conducted at a temperature of about 23°C, and the pH of the working volume was maintained at 6.5 $\pm$ 0.1 by automatic pH control with 1N HNO_3 and 0.5N NaOH.

P. aeruginosa was added to the medium to form a culture. Sodium nitrate ($NaNO_3$) was included in the initial culture medium at a concentration of 0.5 g/liter of NO_3^--N. The sodium nitrate was added periodically throughout the test period to maintain the concentration of NO_3^--N at about 0.1 to about 0.5 g/liter. 16 g/liter of palmitic acid was added to the culture medium as the carbon substrate. The culture medium was allowed to incubate for about 500 hours at the experimental conditions described hereinabove.

The results demonstrate that rhamnolipids can be produced under anaerobic denitrification conditions, without the problems of foaming and oxygen limitation. The present invention provides a process for the production of biological products, wherein a desired amount of a surfactant is added to the culture medium to facilitate the mass transfer of the otherwise poorly soluble or insoluble carbon substrate into the culture medium. The culture is allowed to incubate for a time sufficient to produce a desired quantity of a biological product.

It has been found that the addition of the surfactant to the culture medium facilitates the dispersion or solubilization of the carbon source into the culture medium. Furthermore, the addition of the surfactant to the culture medium assists in the penetration of the carbon source through the cell wall of the microorganism. The amount of surfactant to be added to the culture medium is from about 0.01 g/L to about 10 g/L, preferably from about 0.05 g/L to about 1 g/L, and most preferably from about 0.1 g/L to about 0.5 g/L. The results indicate that rhamnolipids are produced by *Pseudomonas aeruginosa* under denitrifying anaerobic conditions. The rate of rhamnolipid production by anaerobic denitrification is about 2 milligrams of rhamnolipids/gram of cell protein/hour.

Biosurfactants, such as rhamnolipids, are extremely effective in emulsifying and solubilizing hydrocarbons and, therefore, are quite useful in oil recovery processes and mobilizing non-aqueous phase liquid contaminants in soils and aquifers. Rhamnolipids, because of their antibacterial, antiviral, antifungal, and mycoplasmacidal properties, also find potential use in the pesticide applications. In addition, rhamnolipids have been implicated as an additive to concrete formulations to increase the strength of concrete, thus reducing the potential for concrete damage.

Rhamnolipids have particular application in industrial petroleum processes, including emulsification and demulsification, separation, formation of low viscosity emulsion products to transport heavy crudes, emulsion washing, formation of slurries, corrosion inhibition, enhancement of oil recovery and promotion of hydrocarbon biodegradation.

Biosurfactants, such as rhamnolipids, are particularly useful in the cosmetic or personal hygiene industry, because of their low toxicity, excellent moisturizing properties and compatibility with mammalian skin.

The rhamnolipids produced according to the method of the present invention can also be used as a source of rhamnose sugar. The isolated rhamnolipids are hydrolyzed to produce a mixture of rhamnose sugar and beta-hydroxydecanoic acid. The rhamnose is easily separated from the beta-hydroxydecanoic acid. The rhamnose can be used as a fine chemical in many industrial and scientific applications.

According to the present invention, much larger cell concentrations may be employed to give a higher volumetric productivity and product concentrations for more economical product recovery and purification.

The use of the methods of the present invention in biological processes effectively circumvents the limitations of oxygen supply and foam-generation problems that are traditionally associated with aerobic production of biological products such as biosurfactants.

The extremely soluble alternative oxidants can be easily supplied to meet the respiration needs of high cell concentrations and, consequently, achieve very high

process productivity, without the need for vigorous agitation of the culture medium within the reactor.

It has also been demonstrated that the utilization of the methods of the present invention in biological processes is beneficial to the production of biological materials that are sensitive to the presence of molecular oxygen.

Based upon the foregoing disclosure and description, it should now be apparent that the use of the described methods herein will carry out the objects set forth hereinabove. It is, therefore, to be understood that any variations evident fall within the scope of the claimed invention, and the selection of specific carbon sources, culture media, alternate oxidant sources, limiting nutrients, pH and temperature conditions, and selection of microorganism can be determined without departing from the spirit of the invention herein disclosed and described. Thus, the scope of the invention shall include all modifications and variations that may fall within the scope of the claims.

WE CLAIM:

1. A process for the production of biological products by microorganisms comprising the steps of:

selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium;

supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration;

maintaining the culture medium at a desired pH and temperature; and

allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

2. The process of claim 1, further comprising the steps of isolating and recovering said biological product from said culture media.

3. The process of claim 1, wherein the microorganism is selected from the group consisting of bacteria, yeasts, molds and archaea.

4. The process of claim 3, wherein the microorganism is a bacteria.

5. The process of claim 4, wherein bacteria is a facultative aerobe.

6. The process of claim 5, wherein the facultative aerobe is from a genus selected from the group consisting of *Pseudomonas, Paracoccus, Micrococcus, Klebsiella, Escherichia, Acidianus, Campylobacter, Wolinella, Desulfovibrio, Clostridium,* and *Proteus*.

7. The process of claim 6, wherein the genus is *Pseudomonas*.

8. The process of claim 7, wherein the species of the genus *Pseudomonas* is selected from the group consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas cruciviae, Pseudomonas boreopolis* and *Pseudomonas oleovorans*.

9. The process of claim 8, wherein the species of *Pseudomonas* is *Pseudomonas aeruginosa.*

10. The process of claim 1, wherein the carbon substrate is selected from the group consisting of fatty acids, glycerol, low molecular weight acids, carbohydrates, yeast extract, peptone and vegetable oil.

11. The process of claim 10, wherein the fatty acids are selected from the group consisting of palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, and linolenic acid.

12. The process of claim 11, wherein the fatty acid is palmitic acid.

13. The process of claim 10, wherein the vegetable oil is selected from the group consisting of corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil and sunflower oil.

14. The process of claim 13, wherein the vegetable oil is corn oil.

15. The process of claim 10, wherein the carbohydrate is glucose.

16. The process of claim 10, wherein the low molecular weight acid is selected from the group consisting of malate, acetate and pyruvate.

17. The process of claim 1, wherein the alternative oxidant source is selected from the group consisting of nitrates, nitrites, sulfates, sulfites, carbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

18. The process of claim 17, wherein the alternative oxidant source is a nitrate.

19. The process of claim 18, wherein the nitrate is selected from the group consisting of sodium nitrate, potassium nitrate, calcium nitrate, magnesium nitrate, ammonium nitrate, and nitric acid.

20. The process of claim 19, wherein the nitrate is sodium nitrate.

21. The process of claim 17, wherein the nitrites are selected from the group consisting of sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrite, and nitrous acid.

22. The process of claim 17, wherein the sulfates are selected from the group consisting of sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, and dilute sulfuric acid.

23. The process of claim 17, wherein the sulfites are selected from the group consisting of calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite and manganese sulfite.

24. The process of claim 17, wherein the carbonates are selected from the group consisting of calcium carbonate, sodium carbonate, and potassium carbonate.

25. The process of claim 17, wherein the bicarbonates are selected from the group consisting of calcium bicarbonate, sodium bicarbonate, and potassium bicarbonate.

26. The process of claim 17, wherein the fumarates are selected from the group consisting of disodium fumarate, sodium fumarate, dipotassium fumarate, potassium fumarate, and fumaric acid.

27. The process of claim 1, further comprising the step of adding a sufficient amount of a surfactant to said culture medium to facilitate the mass transfer of said carbon substrate into said culture medium.

28. The process of claim 1, further comprising the step of limiting an essential growth nutrient from the culture medium.

29. The process of claim 28, wherein the essential growth nutrient is selected from the group consisting of phosphorous, nitrogen, sulfur, calcium, magnesium and iron.

30. The process of claim 29, wherein the essential growth nutrient is phosphorous.

31. The process of claim 1, wherein said cellular concentration of said microorganism is from about 0.1 g/L to about 50 g/L.

32. The process of claim 1, wherein the concentration of the alternative oxidant source in the culture medium is in the range of from about 0.01 to about 10 g/L.

33. The process of claim 1, wherein the culture is maintained in a temperature range of about 20°C to about 40 °C.

34. The process of claim 1, wherein the culture is maintained in a pH range of about 4 to about 9.

35. A process for the preparation of biological products under anaerobic respiring conditions comprising:

selecting a microorganism that is capable of utilizing an alternative oxidant source other than oxygen for cellular respiration under anaerobic conditions;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

supplying an alternative oxidant source other than oxygen to the culture medium;

maintaining the culture medium at a desired pH and temperature; and

allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

36. The process of claim 35, further comprising the steps of isolating and recovering said biological product from said culture media.

37. The process of claim 35, wherein the microorganism is selected from the group consisting of bacteria, yeasts, mold and archaea.

38. The process of claim 37, wherein the microorganism is a bacteria.

39. The process of claim 38, wherein the bacteria is selected from the group consisting of obligate anaerobes and facultative aerobes.

40. The process of claim 39, wherein the obligate anaerobe is from a genus selected from the group consisting of *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfobulbus, Desulfococcus, Desulfobacter, Desulfosarcine, Desulfonema, Desulfurmonas, Thermoproteus, Pyrococcus, Thermococcus*, and *Shewanella*.

41. The process of claim 40, wherein the facultative aerobe is from a genus selected from the group consisting of *Pseudomonas, Paracoccus, Micrococcus, Klebsiella, Escherichia, Acidianus, Campylobacter, Wolinella, Desulfovibrio, Clostridium*, and *Proteus*.

42. The process of claim 41, wherein the genus is *Pseudomonas*.

43. The process of claim 42, wherein the species of the genus *Pseudomonas* is selected from the group consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas cruciviae, Pseudomonas boreopolis* and *Pseudomonas oleovorans*.

44. The process of claim 43, wherein the species of *Pseudomonas* is *Pseudomonas aeruginosa*.

45. The process of claim 35, wherein the carbon substrate is selected from the group consisting of fatty acids, glycerol, low molecular weight acids, carbohydrates, yeast extract, peptone and vegetable oil.

46. The process of claim 45, wherein the fatty acids are selected from the group consisting of palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, and linolenic acid.

47. The process of claim 46, wherein the fatty acid is palmitic acid.

48. The process of claim 45, wherein the vegetable oil is selected from the group consisting of corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil and sunflower oil.

49. The process of claim 48, wherein the vegetable oil is corn oil.

50. The process of claim 45, wherein the carbohydrate is glucose.

51. The process of claim 45, wherein the low molecular weight acid is selected from the group consisting of malate, acetate and pyruvate.

52. The process of claim 35, wherein the alternative oxidant is selected from the group consisting of nitrates, nitrites, sulfates, sulfites, carbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

53. The process of claim 52, wherein the alternative oxidant source is a nitrate.

54. The process of claim 53, wherein the nitrate is selected from the group consisting of sodium nitrate, potassium nitrate, calcium nitrate, magnesium nitrate, ammonium nitrate, and nitric acid.

55. The process of claim 54, wherein the nitrate is sodium nitrate.

56. The process of claim 35, wherein the nitrites are selected from the group consisting of sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrite, and nitrous acid.

57. The process of claim 35, wherein the sulfates are selected from the group consisting of sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, and dilute sulfuric acid.

58. The process of claim 35, wherein the sulfites are selected from the group consisting of calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium

sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite and manganese sulfite.

59. The process of claim 35, wherein the carbonates are selected from the group consisting of calcium carbonate, sodium carbonate, and potassium carbonate.

60. The process of claim 35, wherein the bicarbonates are selected from the group consisting of calcium bicarbonate, sodium bicarbonate, and potassium bicarbonate.

61. The process of claim 35, wherein the fumarates are selected from the group consisting of disodium fumarate, sodium fumarate, dipotassium fumarate, potassium fumarate, and fumaric acid.

62. The process of claim 35, further comprising the step of adding a sufficient amount of a surfactant to said culture medium to facilitate the mass transfer of said carbon substrate into said culture medium.

63. The process of claim 35, further comprising the step of limiting an essential growth nutrient from the culture medium.

64. The process of claim 63, wherein the essential growth nutrient is selected form the group consisting of phosphorous, nitrogen, sulfur, calcium, magnesium and iron.

65. The process of claim 64, wherein the essential growth nutrient is phosphorous.

67. The process of claim 35, wherein said cellular concentration of the microorganism in the culture medium is in the range of from about 0.1 g/L to about 50 g/L.

68. The process of claim 35, wherein the concentration of the alternative oxidant source in the culture medium is in the range of from about 0.01 to about 10 g/L.

69. The process of claim 35, wherein the culture is maintained in a temperature range of about 20°C to about 40 °C.

70. The process of claim 35, wherein the culture is maintained in a pH range of about 4 to about 9.

71. A process for increasing concentration of microorganisms in a defined medium comprising the steps of:

selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium;

supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen

for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration;

maintaining the culture medium at a desired pH and temperature; and.

allowing the culture medium to incubate for a time sufficient to produce a desired concentration of microorganisms within the culture medium.

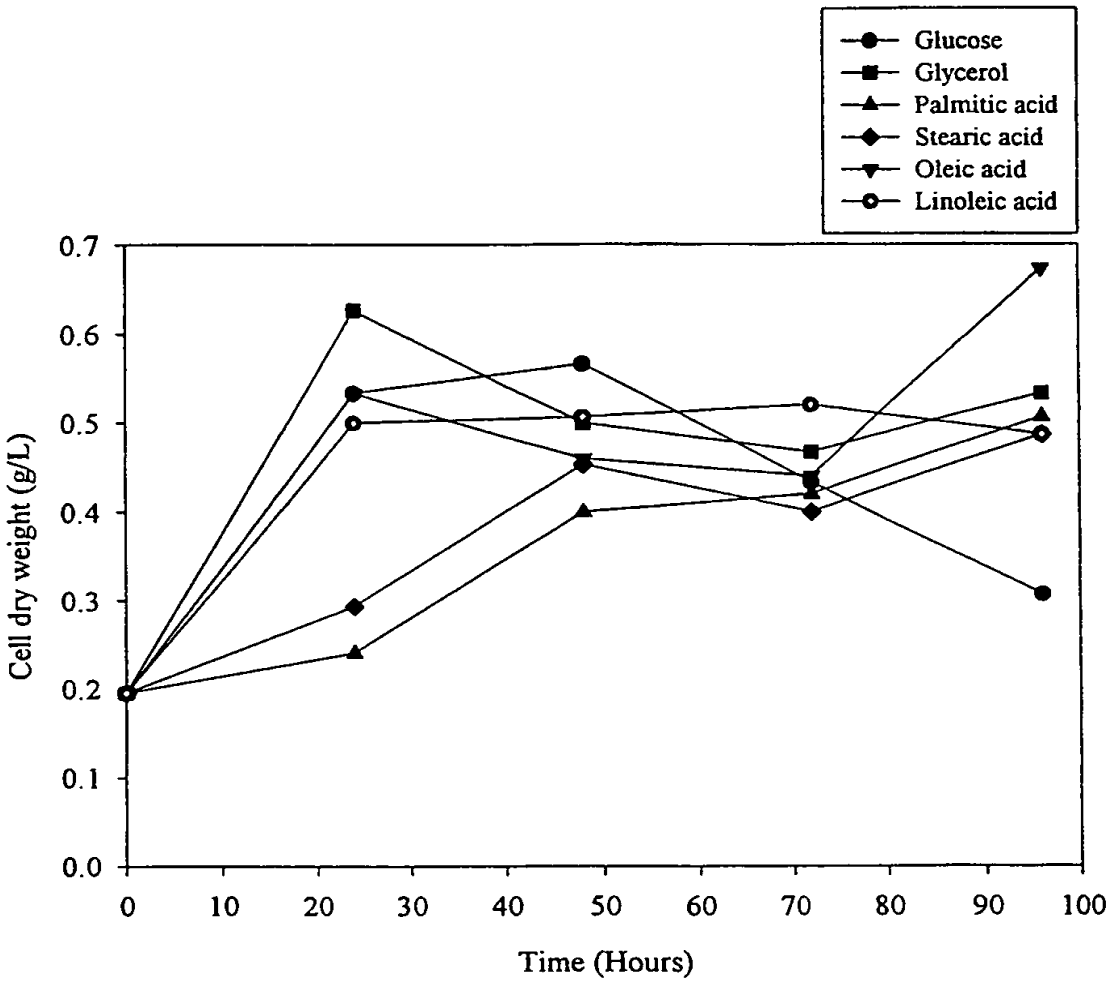
Glucose
Glycerol
Palmitic acid
Stearic acid
Oleic acid
Linoleic acid
Cell dry weight (g/L)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
10
20
30
40
50
60
70
80
90
100
Time (Hours)

Figure 1

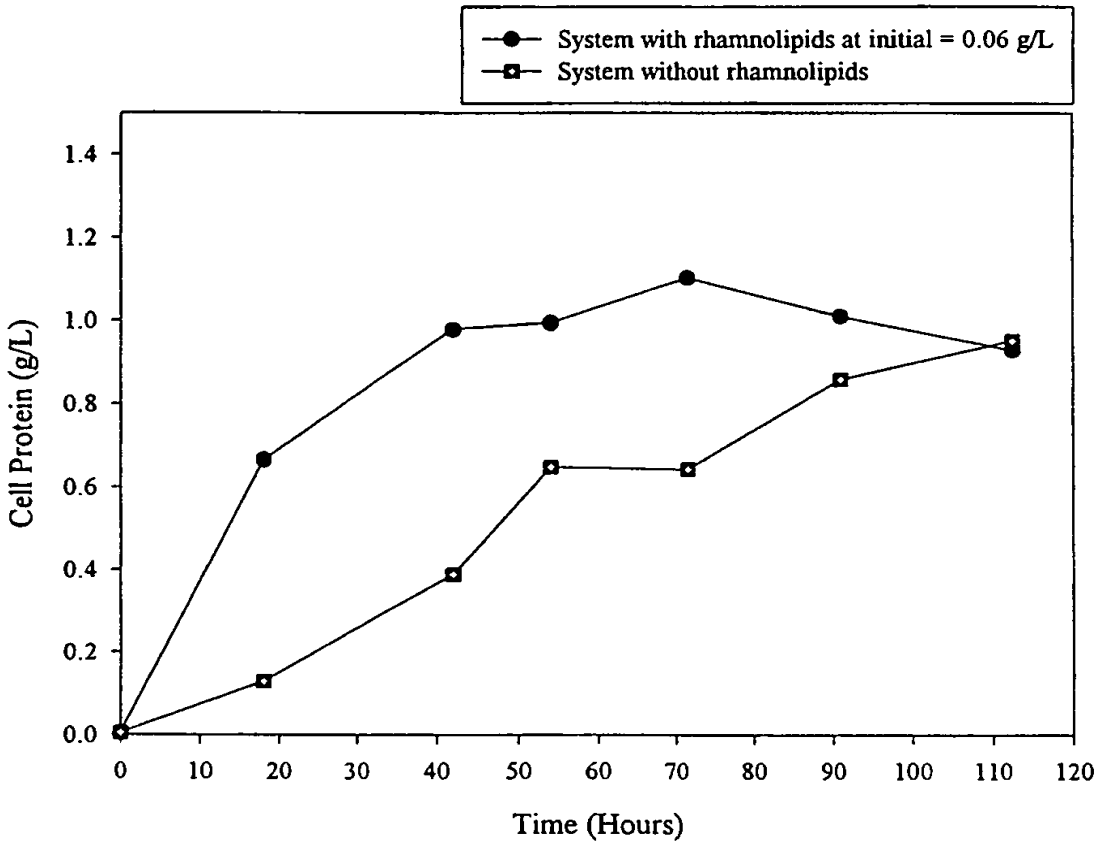
System with rhamnolipids at initial = 0.06 g/L
System without rhamnolipids
Cell Protein (g/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (Hours)

Figure 2

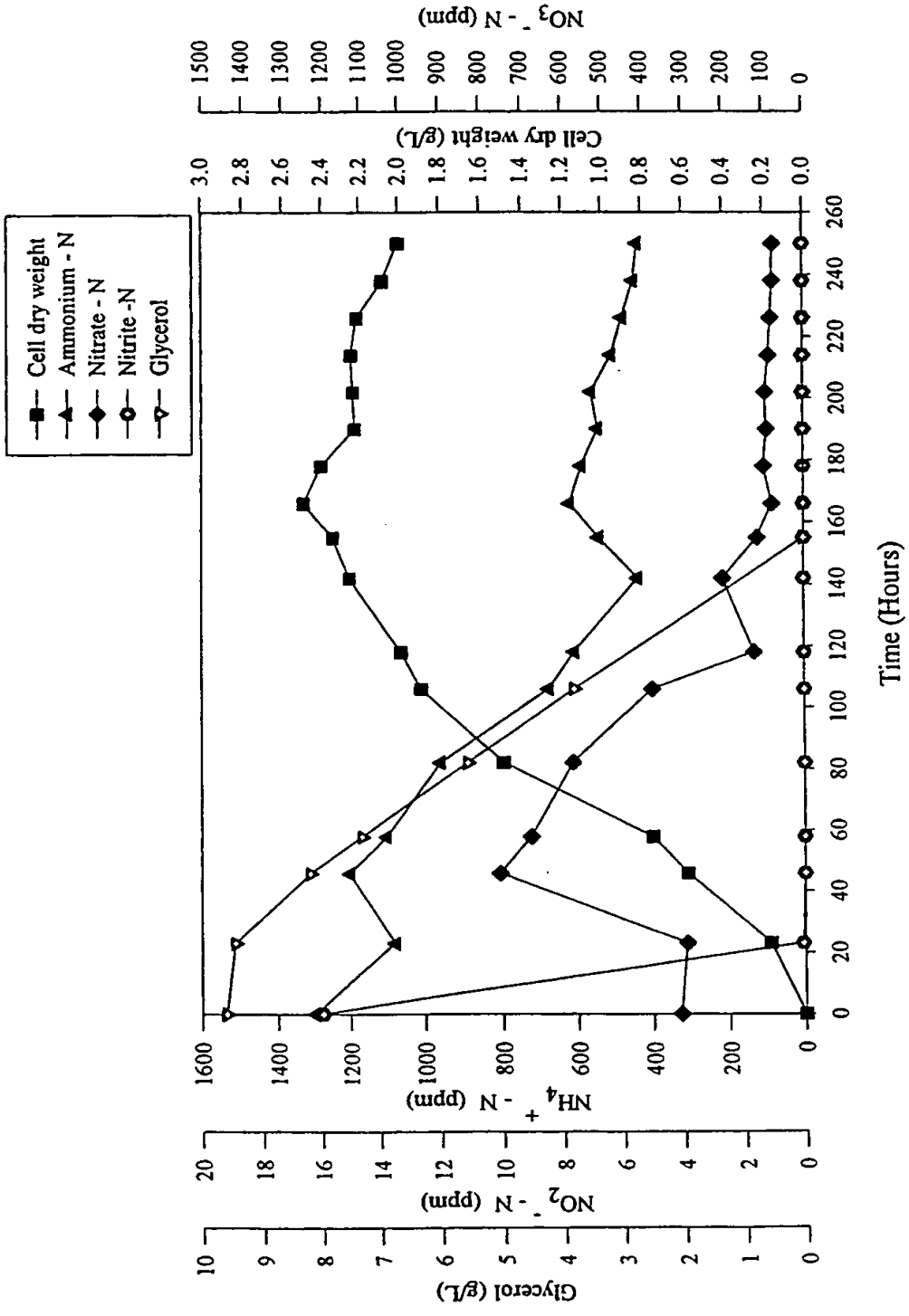
Cell dry weight
Ammonium - N
Nitrate - N
Nitrite -N
Glycerol
Time (Hours)
Glycerol (g/L)
NO_2^- - N (ppm)
NH_4^+ - N (ppm)
Cell dry weight (g/L)
NO_3^- - N (ppm)

Figure 3

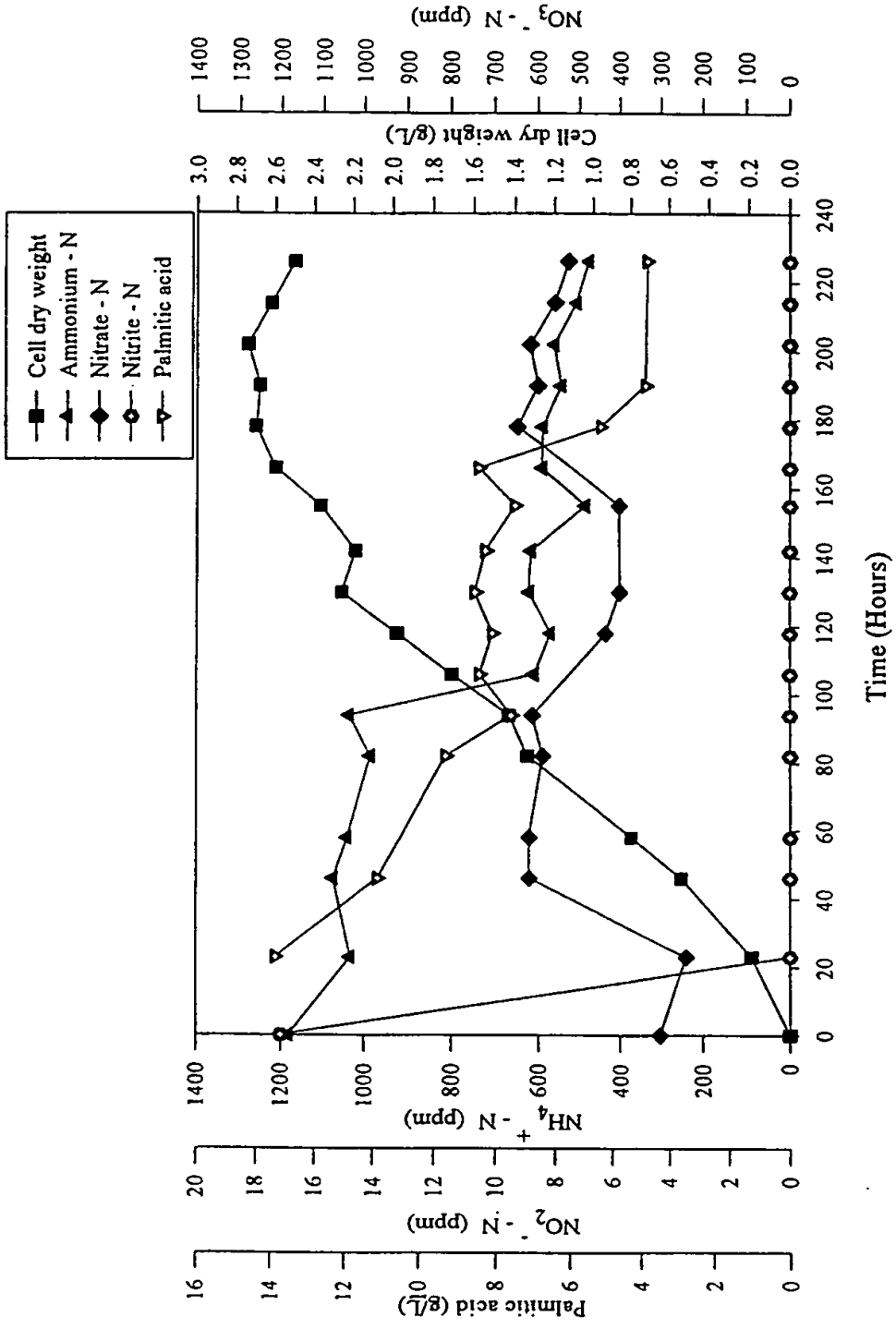
Cell dry weight
Ammonium - N
Nitrate - N
Nitrite - N
Palmitic acid
Palmitic acid (g/L)
NO_2^- - N (ppm)
NH_4^+ - N (ppm)
Cell dry weight (g/L)
NO_3^- - N (ppm)
Time (Hours)

Figure 4

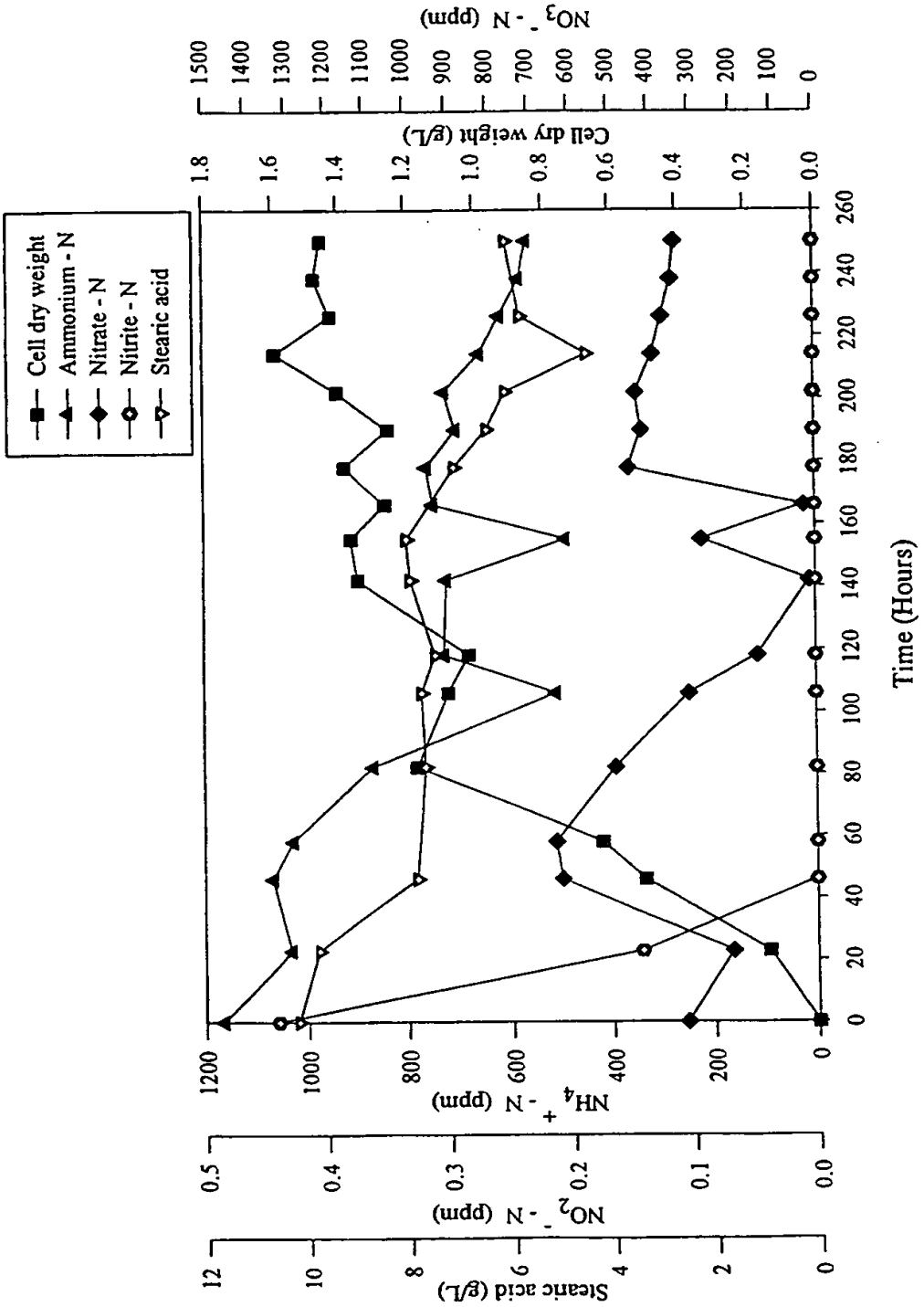
Cell dry weight
Ammonium - N
Nitrate - N
Nitrite - N
Stearic acid
Time (Hours)
Stearic acid (g/L)
NO_2^- - N (ppm)
NH_4^+ - N (ppm)
Cell dry weight (g/L)
NO_3^- - N (ppm)

Figure 5

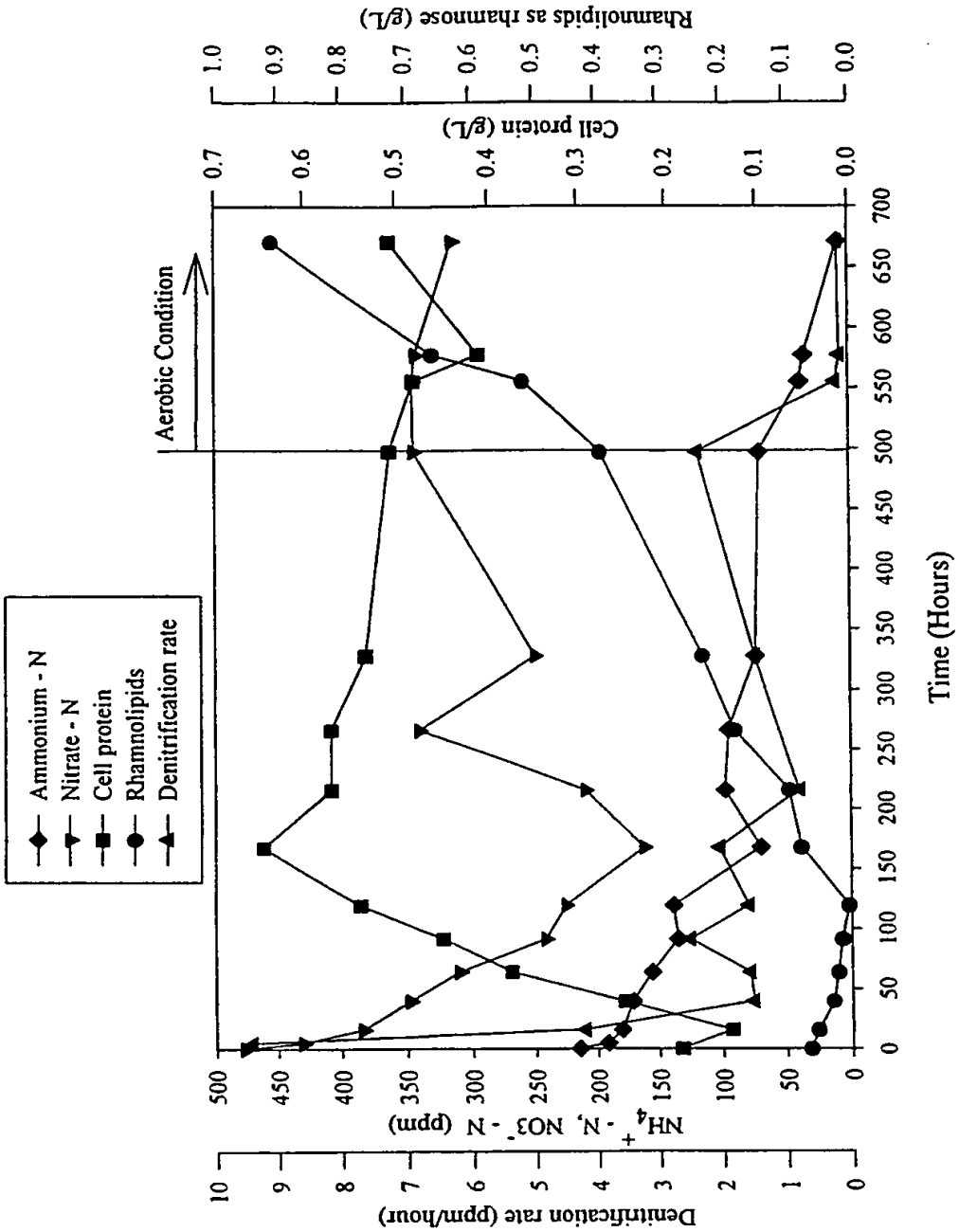
Ammonium - N
Nitrate - N
Cell protein
Rhamnolipids
Denitrification rate
Aerobic Condition
Time (Hours)
Denitrification rate (ppm/hour)
NH4+ - N, NO3- - N (ppm)
Cell protein (g/L)
Rhamnolipids as rhamnose (g/L)

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 1/00, 39/00, 19/02. 19/44; C12N 1/20
US CL :435/41, 42, 74, 105, 253.3; 536/4.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/41, 42, 74, 105, 253.3; 536/4.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,501,966 A (GIANI ET AL) 26 MARCH 1996 (26/03/1996), see entire document, especially columns 3-9.	1-20,22-25, 27-38,41- 71 ---------- 21, 26, 39 and 40
Y	VARMA AMIT et al. Stoichiometric Flux Balance Models Quantitatively Predict Growth and Metabolic By-Product Secretion in Wild-Type Escherichia coli W3110. Applied and Environmental Microbiology.October 1994, Vol. 60, No. 10, pages 3724-3731, see entire document.	1-71

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 11 FEBRUARY 2000	Date of mailing of the international search report 14 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-1235

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ATLAS RONALD. Hand Book of Microbiological Media. Ann Arbor: CRC press. 1993, pages 290-301, see entire document.	21,26,39 and 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN, WEST, HCAPLUS, BIOSIS, MEDLINE, EMBASE, LIFESCI, SCISEARCH, WPIDS.
search terms: anaerobic, aerobic, facultative aerobe , pseudomonas, obligate anaerobe, desulfomonas, desulfobacter, culture, media.